# Transport processes in Sulfolobales

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Cover design and image: Nuan Yang
Layout and printing: Jolanda Brons;
CPI, Koninkijke Wohrmann, the Netherlands
ISBN: 978-90-367-7685-1 (book)
ISBN: 978-90-367-7684-4 (electronic version)
The work described in this book was carried out in the Department of Molecular Microbiology of the Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, the Netherlands and was financially supported by the China Scholarship Council.

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# Transport Processes in *Sulfolobales*

Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
vrijdag 27 maart 2015
om 14.30 uur

door

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geboren op 29 july 1982
te Beijing, China

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|---|---|
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# INTRODUCTION

# TRANSPORTERS IN THE THIRD DOMAIN OF LIFE

## ARCHAEA

The first archaeon, *Sulfolobus acidocaldarius* (Brock *et al.,* 1972) was isolated in 1972, and at that time considered as a thermo-acidophilic bacterium (Shivvers and Brock, 1973). When Carl Woese examined the rRNA as a phylogenetic marker (Woese *et al.,* 1990; Woese and Fox, 1997) it became apparent that the archaea represent a unique branch in the evolutionary tree of life together with the eukaryotes and bacteria (Woese *et al.,* 1990; Woese and Fox, 1997; Garrett and Klenk, 2007) (Fig. 1). This was further confirmed by studies of the archaeal biochemistry and molecular biology showing unique features such as the ether lipid based membranes. Since that time, the interest in this domain of life has increased because it encompasses a wide metabolic diversity often associated with life at extreme environments.

Archaea are ubiquitous and well represented in diverse ecological niches. They were found to adapted to a range of habitats and life styles (Barns *et al.,* 1994; Hershberger *et al.,* 1996; Bintrim *et al.,* 1997; Jurgens *et al.,* 1997; Buckley *et al.,* 1998; Reysenbach *et al.,* 2000; Karner *et al.,* 2001; Ochsenreiter *et al.,* 2002; Church *et al.,* 2003; Ochsenreiter *et al.,* 2003; Futterer *et al.,* 2004; Lepp *et al.,* 2004). Best known are the extremophilic archaea that thrive at high temperature (hyperthermophiles), extreme pH values (acidophiles and alkalophiles) and high osmotic pressure (halophiles), but also the methanogens that are strictly anaerobic and responsible for a major share of the biogenic methane production at this globe (Pikuta *et al.,* 2007). However, archaea are not only found in extreme environments but widespread over the world, and present at 'common' ecological niches that are not considered as extreme.

Archaea fulfill a main role in several biogeochemical cycles of the biosphere. In general, the study of this domain of life will increase our understanding of life thriving in extreme environments, processes of fundamental biogeochemistry, the origin of life, and fundamental aspects of their unique features. Archaea share similar characteristics either with bacteria or eukaryotes (Olsen and Woese, 1977; Bernander 2000), whereas they also have unique features (Albers *et al.,* 2011). They are unicellular prokaryotes that lack membrane-surrounded organelles and a nucleus. The similarities to bacteria are most obvious from their morphology, metabolic traits and life style including the presence of small single circular chromosomes and genes that are organized in operons (Koonin *et al.,* 1997). The basal cellular machinery of archaea, however, shows more similarity to processes in eukaryotes rather than bacteria. Despite the fact that archaeal cells lack a nucleus, the components of the replication, transcription and translation machinery are homologous to the corresponding eukaryotic counterparts (Keeling and Doolittle, 1995; Langer *et al.,* 1995; Dennis 1997; Bell and Jackson, 2001; Grabowski and Kelman, 2003; Maupin-Furlow *et al.,* 2006). During the last decade, the genomes of many archaea have been sequenced and annotated. Most ORFs show homology either to bacterial or eukaryal genes, but about a third of the genes is unique to archaea. Using such similarities and differences, various theories have been developed on the evolutionary origin of the three domains of life and in particular LUCA, which is the Last Universal Common Ancestor. Studies on archaea in general are focused on the molecular basis of life under extreme conditions, as well as unique features of their carbon- and nitrogen metabolism. Archaea lack a peptidoglycan wall or substitute polymer, and rely on a highly glycosylated S-layer (Eichler 2003) that forms the most outer surface of the cell envelop providing protection and mechanical support (Engelhardt 2007). Importantly, the

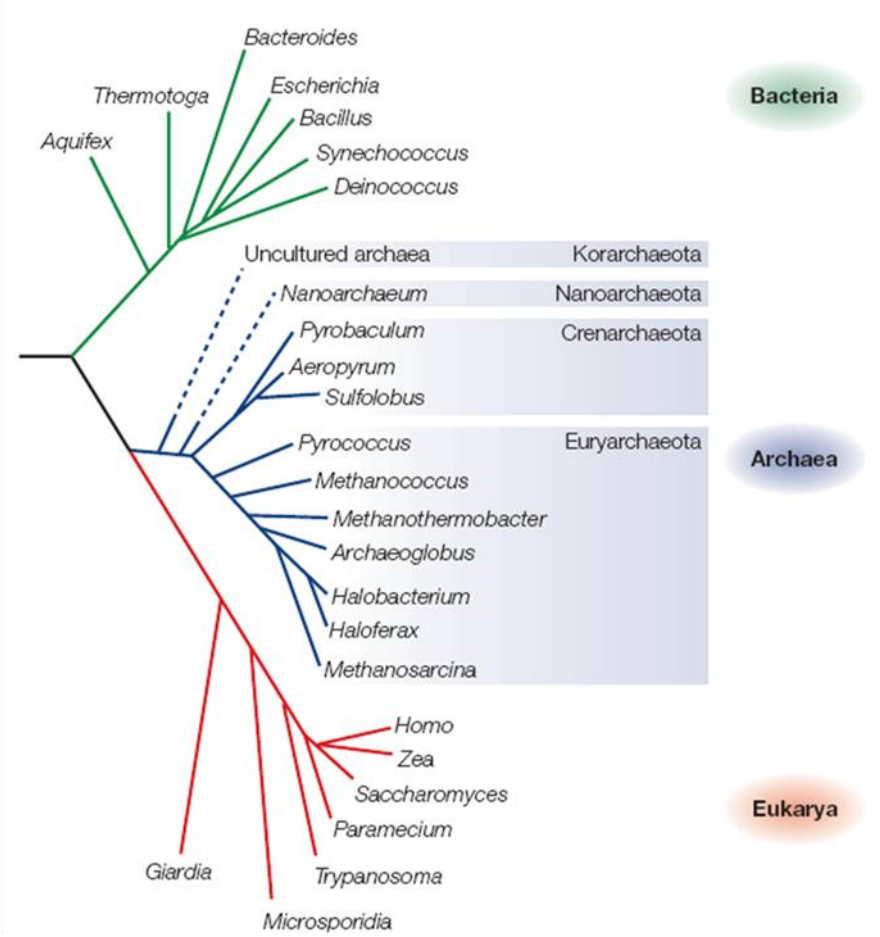


**Fig. 1** Phylogenetic tree of three domains of life based on rRNA sequence. Symbols represent: Green (Bacteria), Blue (Archaea) and Red (Eukarya). Major phyla (kingdoms) within the Archaeal domain are indicated. (Allers *et al*., 2005)

archaeal membrane consists of unique phospholipids in which the glycerol backbone is ether linked to isoprenoid chains at the sn-2, 3 position rather than the ester-based lipids typical for eukaryotes and bacteria. Many archaea contain in addition to such diether lipids, tetraether lipids that are membrane spanning. It is believed that the unique composition of the archaeal membrane also is one of the key adaptations that allow them to thrive under extreme growth conditions, such as high temperature and low pH. On the other hand, some key biological features are eukaryote like, for instance, the archaeal DNA replication machinery. Most studies on the archaeal cell cycle have been reported in Crenarchaea. This in particular concerns the genus *Sulfolobu*s (Lundgren *et al.,* 2008). Flow cytometry studies show that crenarchaeal cells contain two copies of their genome during the exponential growth phase indicative of a long post DNA replication period, while DNA replication takes round 15-30% of the cell cycle (Lundgren *et al.,* 2008).

The phylogenetic classification of archaea is still contentious and rapidly changing. Three main branches have been recognized at the phylum level in the archaeal domain: Euryarchaeota, Crenarchaeota and Thaumarchaeota (Woese *et al.,* 1990; Barns *et al.,* 1996; Huber *et al.,* 2002; Brochier-Armanet *et al.,* 2008; Elkins *et al.,* 2009; Guy and Ettema, 2011). It is a little harder to indicate in what they differ, this is mostly phylogenetically, most of the culturable and well-investigated species are members of the Euryarchaeota and Crenarchaeota. The Euryarchaeota comprise methanogens, halophiles, (hyper-, acido-) thermophiles (Fiala and Stetter,

1986; Schleper *et al.,* 1995). Crenarchaeota mostly consists of thermophilic organisms, such as *Pyrolobus fumarii* (Blöchl *et al.,* 1997) and the intensively studied extreme thermoacidophile *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* (Brock *et al.,* 1972; Zillig *et al.,* 1980). The Thaumarchaeota group consists amongst others of mesophilic archaea for instance living in marine environments. Other groups exist such as the Korarchaea (Barns *et al.,* 1996; Elkins *et al.,* 2008) and Nanoarchaea (Huber *et al.,* 2002). However, the taxonomic position of these phyla is still under debate. Korarchaea share features of Euryarchaeota and Crenarchaeota, but seem more affiliate to Crenarchaeota (Brochier *et al.,* 2005; Elkins *et al.,* 2008). Other recently detected species of archaea are only distantly related to any of these groups, such as the Archaeal Richmond Mine Acidophilic Nano-organisms (ARMAN), which were discovered in 2006 (Baker *et al.,* 2006) and are some of the smallest organisms known (Baker *et al.,* 2010).

Thermophiles are capable to thrive at temperatures above 45°C, while the hyperthermophiles sustain growth over 80°C up to 120°C for instance in hydrothermal ecosystems. These habitats exist both as terrestrial and oceanic systems and are rich in nutrients allowing a chemolithotrophic mode of energy conservation. It is of interest to note that thermophiles are present in both the Euryarchaeota and Crenarchaeota, and this had led to the suggestion that there is a thermophilic origin for all archaea (Woese 1987). Thermophilic archaea are of special interest to biochemists and structural biologists, owing to the presence of thermo-stable enzymes that are often more amendable to protein crystallization. Since the discovery of *S. acidocaldarius*, over 90 hyperthermophilic species have been discovered (Stetter 2006a, 2006b, 2013). Most of them belong to the domain of archaea, but some hyperthermophilic bacteria have also been characterized.

## CRENACHAEOTA MODEL ORGANISMS – *SULFOLOBALES*

*Sulfolobus* species are considered as model organisms for the phylum Crenarchaeota and intensively studied with respect to the mechanisms of transcription, translation, and replication, DNA repair, cell division, RNA processing, metabolism, and many other cellular processes. So far, they are the only representatives and genetically tractable members of the Crenarchaeota that are amenable for genetic manipulation. Various genetic tools are now available and their application has led to important new discoveries.

*S. acidocaldarius* was described by Thomas Brock in 1972 and isolated from a hydrothermal hot spring in Yellowstone National Park (Brock *et al.,* 1972). Later on, different members of the *Sulfolobales* were isolated such as *S. solfataricus* (Pozzuoli, Italy; Zillig *et al.,* 1980) and *S. tokodaii* (Japan; Suzuki *et al.,* 2002) mainly from solfataric fields, hot pools, mud pots and soils. In the meantime, *Sulfolobales* have become the most well studied crenarchaeons.

Owing to their sulphur oxidizing capabilities (Sulfo) and lobedappearance (lobus), this genus was named *Sulfolobus*. Sizes of *Sulfolobus* cells typically range from 0.8 to 1.2 µm. They can be grown optimally at temperatures from 60°C to 90°C and thus are considered as hyperthermophiles. They grow at remarkably low pH values from 2 to 4 and thus are also acidophiles (Huber and Stetter, 2000), and therefore termed hyperthermoacidophiles. They are able to produce various cell surface appendages that play an important role in mobility and adherence to diverse surfaces (Zolghadr *et al.,* 2010). In archaea, the archaellum (former: archaeal flagellum) that functionally analogous to the bacterial flagellum is used for motility and immobilization of surfaces such as in biofilm formation.

Full genome sequences have been determined of all commonly used *Sulfolobus* species and

they contain a comparably high AT content (between 63 and 67%). The genome size of *Sulfolobus* ranges from 2 to 3 Mbp. *S. acidocaldarius* has a smaller genome than the other *Sulfolobus* species (Kawarabayasi *et al.,* 2001; She *et al.,* 2001; Reno *et al.,* 2009; Guo *et al.,* 2011), while *S. solfataricus* has the largest genome (She *et al.,* 2001). Several genomes of *S. islandicus* were sequenced (Whitaker *et al.,* 2005) to describe the mechanism of archaeal genome evolution (Reno *et al.,* 2009). In addition, the availability of the various genome sequences has stimulated comparative analysis and the development of genetic tools to construct gene deletion mutants in *S. solfataricus* (Worthington *et al.,* 2003; Szabo *et al.,* 2007), *S. acidocaldarius* (Wagner *et al.,* 2009; Wagner *et al.,* 2012) and *S. islandicus* (Deng *et al.,* 2009). Since only few antibiotics can withstand the thermoacidophilic conditions, the development of genetic system in *Sulfolobus* was dependent on auxotrophic markers. The *pyrE* gene that encodes orotate phosphoribosyl transferase involved in uracil biosynthesis has been deleted and mutated in *S. acidocaldarius*, MR31 (Reilly and Grogan, 2001) strain. Such strains can be used to select for marker-less deletion mutants that are obtained though a homologous recombination double crossover event whereby the uracil autotrophy is used for enrichment of mutants (Wagner *et al.,* 2009; Ellen *et al.,* 2010). Compared to *S. solfataricus*, *S. acidocaldarius* is more readily assessable for this gene deletion methodology making it a prime candidate for genetic studies (Wagner *et al.,* 2009). Meanwhile, viral or shuttle vector dependent protein overexpression systems have been developed in *Sulfolobus* (Berkner *et al.,* 2007; Berkner and Lipps, 2008) and can be applied for protein production and for the introduction of tagged protein variants to ease biochemical analysis. Overall, these developments now make *Sulfolobus* an attractive model system for functional studies.

*S. acidocaldarius* has been used in a number of cell cycle related studies (Lundgren and Bernander, 2005), also making use of protocols for the synchronization of the cell cycle (Hjort and Bernander, 1999; Lundgren *et al.,* 2004; Duggin *et al.,* 2007), extensive microarray data (Andersson *et al.,* 2005) and gene mutation analysis (Berkner *et al.,* 2008). This will also be outlined in more detail in the section on ESCRT and membrane vesicle formation.

## TRANSPORT IN ARCHAEA

In archaea and bacteria, three main groups of solute transport systems are found that are classified based on energy coupling mechanism, namely: (i) ion channels; (ii) secondary transporters which utilize the electrochemical gradient of protons (or sodium ions) to transport substrates across the membrane in symport or antiport with protons (or sodium ions); and (iii) primary transporters, which use the binding and hydrolysis of ATP to drive substrate transport (Fig. 2). In archaea, the ion channels constitute the smallest class of transport mechanisms. More than half of the archaeal transporters belong to the class of secondary transporters, while the remainders are ABC transporter systems. In thermophilic bacteria, this relationship appears inverted and ABC transporter systems are most predominant class. Most of the predicted secondary transporters are putative inorganic ion transporters, while ABC transporters are the more abundant transporters for organic solutes.

### SECONDARY TRANSPORTERS

Secondary transporters usually consist of one polypeptide chain typically with 12 transmembrane domains. They can be discriminated into three classes: (a) symporters, which transport solutes

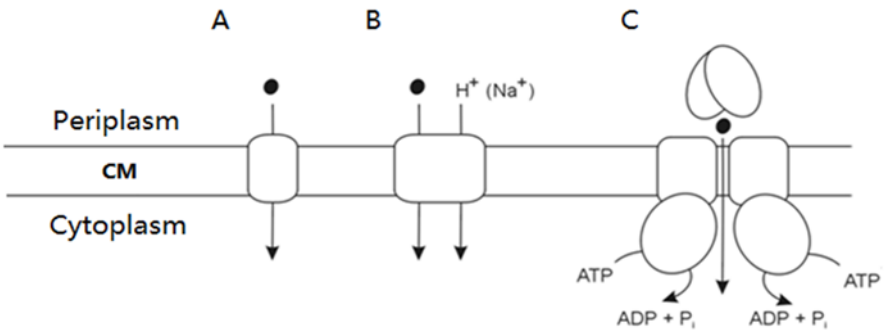


**Fig. 2** Schematic overview of the different solute uptake mechanisms, namely channels (A), secondary transporters (B), and binding-protein-dependent ABC transporter (C), CM, cytoplasmic membrane. (Koning *et al*., 2002)

together with a coupling protons or sodium ions; (b) uniporters, which translocate solutes without coupling ion; and (c) antiporters, which transport substrates/ions in opposite directions.

Interestingly, in acidophilic archaea (*P. torridus, S. solfataricus, S. tokodaii, T. acidophilum and T. volcanicum*), the fraction of secondary transporters is very high, around 70%. The NiCoT ($Ni^{2+}/Co^{2+}$ transporter) and Nramp (metal-ion transporter) family, which are involved in the uptake of heavy metal or iron ions, are exclusively found in acidophiles. They may relate to the high concentrations of heavy metal ions present in acidic environments. Some of these transporters might also be involved in resistance mechanisms such as the energy-dependent excretion of the metal ions from the cell. Although secondary transporters appear an important class in thermophilic organisms, few studies have been performed to investigate their significance. A lactose transporter belongs to the major facilitator family (MFS) was identified in *S. solfataricus* by functionally complementation (Prisco *et al.,* 1985; Bartolucci *et al.,* 2003). The first structure of a putative glutamate transporter was from *P. horikoshii* (Yernool *et al*., 2004).

## ABC TRANSPORTERS

Because of the large transmembrane pH gradient across the membrane, hyperthermoacidophilic archaea like *Sulfolobus* would be suspected to rely on the proton motive force for transport. However, these organisms in particular appear to utilize ABC-type transporters for their survival strategy in the oligotrophic extreme environments. Such systems are equipped with an extracellular substrate binding protein that scavenges the substrates from the nutrient-poor environments. Because of the coupling to ATP hydrolysis, also a high level of accumulation can be reached resulting in high intracellular substrate concentrations. This makes binding protein dependent ABC-type transporters optimally suited for growth under nutrient-poor conditions.

ATP-binding cassette (ABC) transporters are an important class of transport proteins. They are ubiquitous and functionally versatile proteins and constitute one of the largest protein families in nature that are widely distributed in all kingdoms from bacteria, archaea to humans (Holland *et al.,* 2003; Lubelski *et al.,* 2007). They function in a variety of processes such as substrate uptake or export, osmosensing regulation, and antigen processing (Holland and Blight, 1999). ABC transporters typically consist of two characteristic regions: a poorly conserved transmembrane domain (TMD) with 6 transmembrane segments, and a highly conserved nucleotide-binding domain (NBD) (Hollenstein *et al.,* 2007) present in the membrane peripheral ABC subunit. Transporters involved in solute uptake are only found in bacteria and archaea and in general

require an additional extracellular substrate binding protein (Lee *et al.,* 2007). With ABC transporters involved in solute excretion, the TMD and ABC subunits are fused into a single polypeptide, an architecture that corresponds to the so-called "half-transporter". The functional unit consists of either a homo- or hetero-dimer of such "half-transporters". In eukaryotes, these subunits may also be fused into one large polypeptide which is termed "full-transporter" (Kerr 2002) corresponding to two TMD and two ABC domains.

In archaea, the binding-protein-dependent ABC transporters are the best-studied group of transporters. The first archaeal binding-protein-dependent ABC transporter discovered in the hyperthermophilic organism *Thermococcus litoralis* (Xavier *et al.,* 1996), showed a high affinity (nanomolar range) for some sugars (Horlacher *et al.,* 1998). In the meantime, many ABC transporters have been functionally characterized in hyperthermoacidophilic archaea (Albers *et al.,* 2004). The presence of these systems in both archaea and bacteria implies that the basic principles of binding-protein-dependent transport must have originated early in evolution.

**ABC subunit as energy coupling factor for substrate transport**

Archaeal ABC transporters share a number of typical consensus sequence motifs with their bacterial counterparts. The ATP-binding subunit drives substrates transport across the membrane by controlling the opening and closure of the membrane integral transport channel. ABC proteins show a high sequence conservation with in particular the Walker A and B motifs that form the nucleotide binding site (Walker *et al.,* 1982). ATP binding at the Walker A and B sites results in the dimerization of two monomers which is a crucial step in the transport cycle; the Q-loop or linker region, the H motif, and the unique ABC signature motif (LSGGQ) (Boos and Shuman, 1998). The latter has been shown to be critical for dimerization (Moody *et al.,* 2002).

Various archaeal ATP-binding proteins have been functionally characterized and crystallized, i.e., LolD (MJ0769) and LivG (MJ1267) from *M. jannaschii* (Yuan *et al.,* 2001), MalK from the trehalose/maltose transporter of *T. litoralis* (Diederichs *et al.,* 2000) and GlcV from the glucose transporter of *S. solfataricus* (Verdon *et al.,* 2002). The overall structures of these ATP-binding domains are very similar to that of bacterial and eukaryal counterparts. The ATPase subunit interacts with the EAA motif in one of the cytosolic loops of the membrane domain (Dassa and Hofnung, 1985; Mourez *et al.,* 1997). The latter forms a homodimer or heterodimer and functionally contacts both the ABC subunit and the substrate binding protein.

**Substrate-binding proteins**

Archaeal substrate binding proteins are structurally and functionally very similar to their bacterial counterparts. They capture substrates at the outer surface of the cell with a very high affinity and subsequently transfer the substrate to the membrane integral permease domain (Borths *et al.,* 2002). This process involves large conformational change both in the transporter as well as in the substrate binding protein. ATP binding and hydrolysis at the ABC domains triggers the release of the substrate from the binding protein. The functional interconnection between the substrate binding protein and ABC subunit for the release of substrate is coupled by membrane permease proteins (Quiocho 1990; Davidson 2002; Chen *et al.,* 2001, 2003). In prokaryotes, binding proteins either diffuse freely in the protein-dense periplasm (Gram-negative), are anchored to the membrane via a fatty acid modification of their amino-terminus (Gram-positive) or are membrane

tethered by means of a hydrophobic transmembrane segment at the N or C-terminus (Archaea) (Fig. 3). This membrane anchoring is likely necessary to prevent that the binding protein is released into the medium, although the S-layer is permeable only for small proteins.

Among the subunits of archaeal ABC transporters, the amino acid sequence variation is the greatest with the substrate-binding proteins (Saurin *et al.,* 1994; Koster 2001). Substrate binding proteins typically consist of two independent globular domains that are linked by a flexible hinge with the ligand-binding domain, which is present at the interfacial cleft between the two domains. Upon binding of the substrate, these two domains come together and enclose the ligand (Quiocho and Ledvina, 1996). Such a mechanism is also called a "venus flytrap". Based upon the number of interdomain connections, substrate-binding proteins can be subdivided into three groups (Quiocho and Ledvina, 1996; Borths *et al.,* 2002; Dwyer and Hellinga, 2004). Class I proteins, such as the maltose-binding protein, and Class II proteins, including ribonucleotide reductase, differ in their substrate binding and release characteristics (Quiocho and Ledvina, 1996). More recently, a third class was identified in which the two main domains are bridged by a single R-helical segment, as exemplified by the vitamin $B_{12}$ binding protein BtuF (Borths *et al.,* 2002; Karpowich *et al.,* 2003) and the iron-hydroxamate binding protein FhuD (Borths *et al.,* 2002) in *E.coli*. Unlike Class I and II substrate binding proteins were the domains have two or three interdomain connections, Class III proteins only have one interdomain connection and undergo relatively small conformational changes (Borths *et al*., 2002; Clarke *et al.,* 2002; Karpowich *et al.,* 2003). They are thought to be more restricted in their movements and possess more rigid interdomain linkers than Class I and II substrate binding proteins.

Several archaeal binding proteins have been characterized in detail and shown to be equipped with an exceptional high binding affinity which can even be in the nanomolar range. In this respect, the kinetics of glucose binding to GlcS in *S. solfataricus* was shown to be extremely pH-dependent with fast kinetics only at low pH (Diederichs *et al.,* 2000; Albers *et al.,* 2001). This appears to be an adaptation to the acidic pH in which these organisms normally thrive. To date, archaeal substrate-binding proteins have mostly been studied in some hyperthermophiles, i.e., *P. furiosus*, *T. litoralis*, and *S. solfataricus*. The crystal structure of the trehalose and maltose-binding protein from *T. litoralis* and *P. furiosus* (Diez *et al.,* 2001; Evdokimov *et al.,* 2001) respectively show the typical organization found also in the bacterial substrate-binding proteins (Quiocho and Ledvina, 1996). Most archaeal binding proteins are glycosylated (Sumper *et al.,* 1990; Hettmann *et al.,* 1998; Erra-Pujada *et al.,* 1999; Greller *et al.,* 2001), and this makes that they can readily be isolated from cell envelope fractions by ConA chromatography (Albers *et al.,* 1999; Elferink *et al.,* 2001; Griller *et al.,* 2001; Koning *et al.,* 2001). Glycosylation may serve to increase the stability at the high growth temperatures. Glycosylation is, however, not necessary for substrate recognition as these proteins can be heterologously expressed in *E.coli* (Horlacher *et al.,* 1998; Koning *et al.,* 2001, 2002) and their substrate-binding activity is not affected by deglycosylation (Albers *et al.,* 1999; Elferink *et al.,* 2001). Like some binding proteins in bacteria, archaeal substrate binding proteins have also been implicated to function as receptors in chemotaxis (Koekova *et al.,* 2002) possibly fulfilling a dual role in uptake and sensing.

**Regulation of binding protein expression**

Since the organic substrate availability in the extreme environment is generally low, hyperthermophilic archaea need to respond quickly to the availability of nutrients. The expression

of ABC-transporters is strongly controlled at the transcriptional, translational and protein level. Transcription of genes can be induced by binding or release of regulator proteins to the target DNA. Expression can also be regulated at the level of mRNA stability or post-translationally where proteins are specifically degraded or (in-) activated by phosphorylation or other modifications. When regulation occurs, this is mostly at the transcriptional level. In *S. solfataricus*, the glucose and trehalose binding proteins appear to be constitutively expressed (Elferink *et al.,* 2001). Cellobiose and maltose binding proteins are slightly upregulated when the cells are grown in the presence of the corresponding sugars, while the arabinose binding protein is strongly

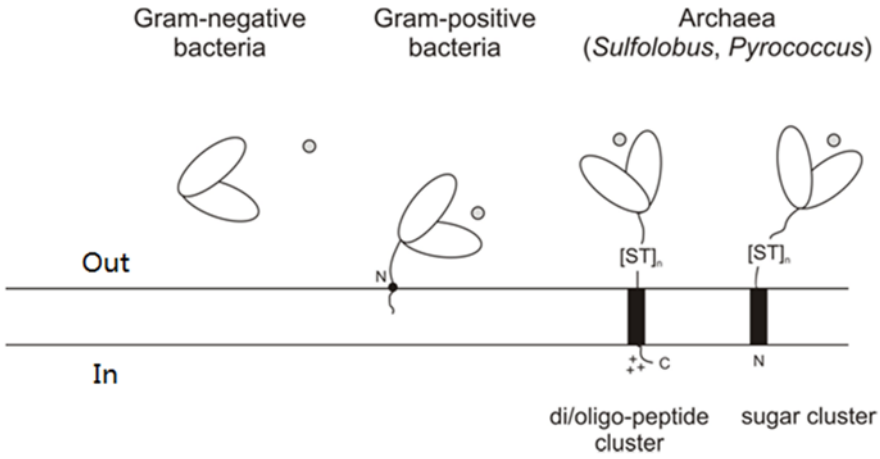


**Fig. 3** Anchoring modes of substrate binding proteins. The binding proteins either move freely in the periplasm (Gram-negative) or are anchored to the membrane via a fatty acyl chain which is attached to the N-terminus (Gram-positive) or a hydrophobic transmembrane segment presented at N/C-terminus (Archaea). (Koning *et al*., 2002)

induced when cells are growing on arabinose. The regulation of the sugar binding proteins of *P. furiosus* has been studied both at the transcriptional and protein level, and expression seems more tightly regulated than in *S. solfataricus*. TrmB is a carbohydrate-specific transcriptional regulator found in all three domains of life (Maruyama *et al*., 2011). The sugar binding domain of TrmB of *T. litoralis* has been crystallized (Krug *et al.,* 2006). It was shown to be involved in the regulation of sugar metabolism, especially for maltose and glucose metabolism (van de Werken *et al.,* 2006; Kanai *et al.,* 2007; Lee *et al.,* 2008). TrmB control the genes encoding the trehalose/maltose ABC transporter in the hyperthermophilic archaeon *T. litoralis* and *P. furiosus* (Lee *et al.,* 2003). In vitro, purified TrmB prevents transcription of the transporter genes but transcriptions restored when trehalose or maltose are added indicating that it behaves as a typical bacterial repressor. Contrary to the Euryarchaeota, TrmB-like transcriptional regulators were found in only three organisms of the phylum Crenarchaeota: *S. acidocaldarius* (Wagner *et al.,* 2013), *Caldivirga maquilingensis* and *Thermophilum pendens* (Maruyama *et al.,* 2011). In *S. acidocaldarius*, the protein was termed MalR but all other Sulfolobus species lack a MalR homolog. The expression of the maltose regulon genes was induced by maltose but required MalR suggesting that in contrast to TrmB homologues, MalR is an activator of the *mal* genes (Wagner *et al*., 2013).

## "TRANSPORTING" VESICLES AND CELL DIVISION

A remarkable but poorly understood phenomenon is the release of membrane vesicles (MVs) from the cell envelop of archaea. This is approved that not only occurs in archaea but in organisms from

all three domains of life (Deatherage *et al.,* 2009), even including Gram-positive bacteria and fungi (Mayer and Gottschalk, 2003; Rodrigues *et al.,* 2007) that contain a thick cell wall. These MVs have been implicated in the intercellular transfer of DNA, lipids or quorum sensing molecules but their exact function has remained obscure. In Gram-negative bacteria this process may be the result of temporal disruptions of the interaction between the outer membrane and the peptidoglycan (Deatherage and Cookson, 2012), it was proposed that vesicles release is part of a stress response. MVs have been isolated from *Halobacteria* and these have been implicated in amino acid uptake (Greene and MacDonald, 1984). The archaeal MVs are coated with S-Layer proteins (Mashburn Warren and Whiteley, 2006), as demonstrated for MVs derived from the thermophilic Euryarchaeon *Aciduliprofundum boonei* (Reysenbach *et al.,* 2006) and Crenarchaeon *Sulfolobus* (Grimm *et al.,* 1998; Prangishvili *et al.,* 2000). Moreover, *Ignicoccus* species produce many periplasmic MVs (Rachel *et al.,* 2002), and these vesicles have been suggested to carry lipids to the intimately associated symbiont Nanoarchaeum that lacks the ability to synthesize lipids. MVs derived from the Euryarchaeon *Thermococcales* bear genomic DNA associated with the surface and were previously thought to be viruses (Soler *et al.,* 2008). The protein composition of MVs is very different from that of the cytoplasmic membrane suggesting that it is selective process. Interesting, in some *Sulfolobus* species, i.e., *S. acidocaldarius* and *S. tokodaii,* antimicrobial proteins termed sulfolobicins, appear to be associated with the MVs and they inhibit the growth of closely related *Sulfolobus* species (Prangishvili *et al.,* 2000; Ellen *et al.,* 2009). The exact relationship between MVs formation and antimicrobial protein secretion is still unknown. Possibly, these antimicrobial proteins associate with the MVs once secreted. Alternatively, they are secreted via the MVs as protected cargo.

Little is known about the mechanisms underlying MV formation. In *Sulfolobus* cells, MVs release into the culture medium is a continuous process (Fig. 4), and presumably associated with membrane scission events that are facilitated by a protein complex that shows homology to the eukaryotic endosomal sorting complex required for transport-III (ESCRT-III) (Ellen *et al.,* 2009) that is further discussed in more detail below. This system has been implicated in cell division in Crenarchaeota (Samson *et al.,* 2008). Interestingly, a proteomic survey of MVs isolated from *S. acidocaldarius* revealed that such vesicles contain two ESCRT-III proteins (termed CdvB1 and CdvB2) and a fragment of a Vps4 homolog (Ellen *et al.,* 2009). This suggests that the *Sulfolobus* ESCRT proteins catalyze the formation of the MVs. However, some other archaea lack the ESCRT-III homologues, but still produce MVs.

## ESCRT SYSTEM AND CELL DIVISION

In Eukarya the ESCRT machinery plays a pivotal role in a wide variety of membrane linked processes such as cytokinesis (Carlton and Martin-Serrano, 2007; Morita *et al.,* 2007; Raiborg and Stenmark, 2009; Morita *et al.,* 2010), vesicles formation (Saksena *et al.,* 2007; Williams and Urbe, 2007; Hurley and Hanson, 2010) and budding of enveloped viruses (Fig. 5) (Morita and Sundquist, 2004; Carlton and Martin-Serrano, 2007; Peel *et al.,* 2011). The ESCRT system has been studied in great detail. The components of the system are organized in several complexes, ESCRT-0, -I, -II and -III, as the AAA+ ATPase Vps4 that acts in concert with the partner proteins Vta1 and Ist1. Some archaea, in particular crenarchaea, contain homologs of the ESCRT-III components and the ATPase Vps4 but homologs of ESCRT-0, -I and -II appear absent (Obita *et al.,* 2007).

The archaeal ESCRT-III components appear to be involved in cell division. However, it should

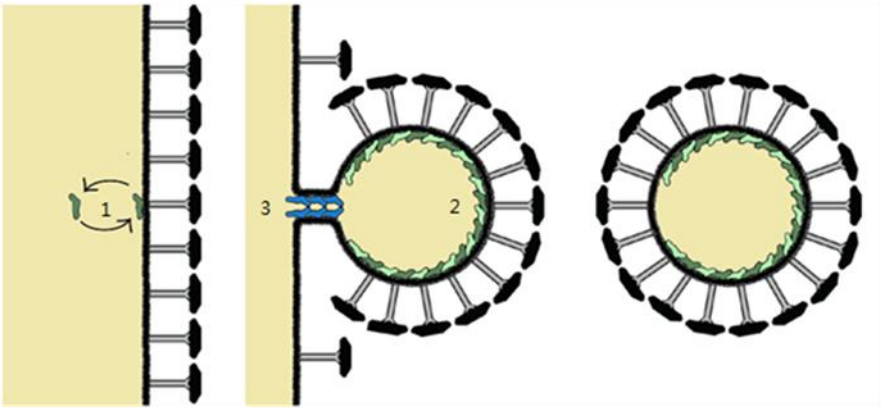


**Fig. 4** Model for vesicle budding in Crenarchaea. Archaeal ESCRT-III like proteins might be involved in **outward budding** of cytoplasmic membrane vesicles resembles **inward budding** of vesicles at endosomal membrane of eukaryotes. (Ellen *et al.,* 2010)

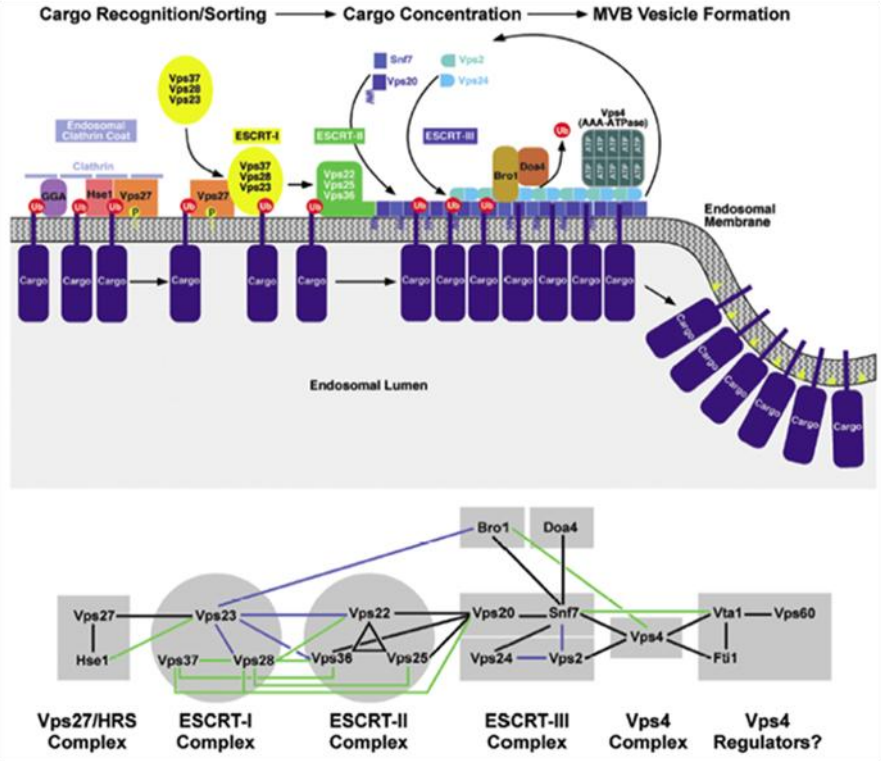


**Fig. 5** Eukaryotic system for recycling of membranes and/or misfolded proteins through inward budding of endosomes and traffic to the lysosome. (Babst 2005)

be emphasized that so far at least three unrelated mechanisms for cell division have been proposed for archaea. Euryarchaeota presumably rely on a FtsZ-based mechanism, a protein that shows structural similarities to eukaryotic tubulin (Wang and Lutkenhaus, 1996; Lowe and Amos, 1998; Poplawski *et al.,* 2000; Makarova and Koonin, 2010), and that in bacteria is a major component of the cell division system. In Korachaea an actin-like system appears to be in place (Makarova *et al.,* 2010; Nunoura *et al.,* 2011), whereas the majority of Crenarchaeota and all Thaumarchaeota seem to utilize the cell division system (Cdv) (Fig. 6) (Lindas *et al., 2008*; Samson *et al.,* 2008) that contains the ESCRT-III like components. In *Sulfolobus* species, the ESCRT-III

homolog (CdvB) and Vps4 (CdvC) protein are encoded in one operon together with a third additional gene which encodes a coiled-coil protein (Lindas *et al.,* 2008; Samson *et al.,* 2008) termed CdvA. The archaeal Vps4 (CdvC) protein contains an N-terminal microtubule-interacting and transport (MIT) domain, which specifically interact with ESCRT-III proteins. An interaction network for the Cdv proteins has been proposed for *Sulfolobales* based on a yeast two-hybrid study and biochemical verification. Similar to the eukaryotic system in which ESCRT-III and Vps4 are interacting partners (Kieffer *et al.,* 2008), CdvB interacts with CdvC (Obita *et al.,* 2007; Samson *et al.,* 2008) and with CdvA (saci_1374) (Moriscot *et al.,* 2011; Samson *et al.,* 2011). Binding between these interacting partners is not mutually exclusive, but there is no direct evidence that CdvA and CdvC interact directly. Besides the Vps4 proximal ESCRT-III protein, *Sulfolobales* contain three additional ESCRT-III homologs that share the similar alpha-helix rich core ESCRT-III fold but that differ in the length and sequence of their C-terminal tail (Samson *et al.,* 2008). These three proteins are able to interact with themselves and each other. In contrast, no interaction was observed with CdvB except for CdvB1 (Samson *et al.,* 2008).

Using synchronized cell cultures, transcription of the ESCRT-III operon (*cdvABC*) shows a characteristic pattern during the cell cycle, with the highest level peaking in dividing cells. Two other ESCRT-III homologs (CdvB1 and CdvB2) also show this characteristic pattern (Lindas *et al.,* 2008), and thus have been implicated in cell division in Crenarchaea.

The Cdv machinery has been characterized at the molecular level in *S. acidocaldarius* (Lindas *et al.,* 2008; Samson *et al.,* 2008; Samson *et al.,* 2011; Dobro *et al.,* 2013), *S. solfataricus* (Samson *et al.,* 2008; Samson *et al.,* 2011) and *Metallosphaera sedula* (Moriscot *et al.,* 2011). The expression of a trans-dominant-negative mutant allele of the Vps4 protein resulted in the formation of large cells with a highly elevated DNA content and also of granular membrane-rich particles devoid of DNA (Lindas *et al.,* 2008). This suggests that expression of the mutant Vps4 interferes with normal cell division. Furthermore, immunolocalization studies provided further evidence that these proteins function in cell division. It revealed a specific localization of ESCRT-III protein which multimerize into a band or ring-like structure at the mid-cell between the segregated nucleoids during constriction (Lindas *et al.,* 2008; Samson *et al.,* 2008). A speculative model for the possible function of the Cdv systems in cell division has been suggested (Fig. 6). CdvA first forms ring-like structures, which may act as a brace to orient the segregating nucleoids at the cell membrane before nucleoid segregation. Next the CdvA ring recruits and facilitates the assembly of the CdvB which in turn recruit the CdvC. CdvA, co-localizing with CdvB, gradually shrinks in size, causing the constriction driving membrane ingression and abscission. As a result, MVs may be pinched off during the constriction as outlined in Fig. 4.

# SCOPE OF THE THESIS

The interest of archaea has continued to grow since it was recognized as one of the three main branches of the evolutionary tree of life together with eukaryotes and bacteria (Woese and Fox, 1977; Woese *et al.,* 1990; Garrett and Klenk, 2007). So far, *Sulfolobus* species are the best-characterized Crenarchaeota of the archaeal domain. They are thermoacidophilic organisms and are able to thrive in extreme hot environments with a very low acidic pH. The optimal growth conditions are around 80°C and pH 2-3. *Sulfolobus* species have a unique cytoplasmic membrane composition with membrane spanning ether lipids (Elferink *et al.,* 1994; Van de Vossenberg *et al.,*

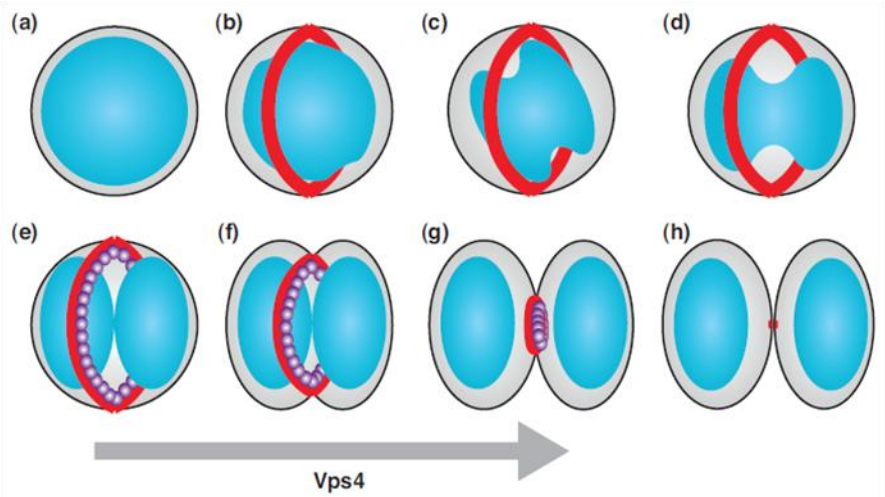


**Fig. 6** (a–h) Speculative model for the assembly of the *Sulfolobus* cell division machinery. A ring of CdvA protein (red) is formed at the cell membrane before nucleoid (blue) segregation. The CdvA ring then recruits and facilitates assembly of CdvB (ESCRT-III) proteins (purple spheres). As the CdvB ring assembles, it recruits the ATPase Vps4 (CdvC) and drives membrane ingression and abscission (e–h). (Rachel 2011)

1998) that owing to their low proton permeability allow cells to maintain a high proton motive force even under the harsh environmental conditions. In addition, other remarkable features occur at the cell envelope. *Sulfolobalen* release membrane vesicles (MVs) from their cell surface but the mechanism underlying this phenomenon is only poorly understood. These membrane vesicles comprise a unique protein composition and it has been suggested that they function in secretion or are leftovers from the cell division process. In this thesis, *Sulfolobus acidocaldarius* was chosen as a model crenarchaea as it is genetically tractable, and was used to study a range of transport processes and cell surface processes such as cell division using gene deletion as major approach.

**Chapter 1** describes an overview of current insights on archaeal transport processes with an emphasis on *Sulfolobales*. It also discusses current insights in the mechanism of cell division.

**Chapter 2** focuses on the cell division system (Cdv) of *Sulfolobales*. This system contains proteins homologous to the eukaryotic ESCRT-III system. Most crenarcheota contain in addition to the CdvB (ESCRT-III, Vps24) proteins, a set of paralogs. These are termed CdvB1-3 in *S. acidocaldarius*. The role of these three proteins was investigated in **Chapter 2** using gene deletions and immunolocalization. Only one of the gene deletion strain (*ΔcdvB3*) showed a severe growth defect with the formation of enlarged cells and a defect in DNA segregation. Remarkably, this is also the only CdvB paralog whose expression is cell cycle related. In the *ΔcdvB3* strain, the key division proteins CdvA and CdvB mislocalize within the cell. It is concluded that CdvB3 in addition to CdvB, fulfills an important role in cell division.

**Chapter 3** describes the identification of an ABC-MDR protein (Saci_2123) that is structurally and functionally related to multidrug transporters and excretes unrelated toxic compounds from the cell. The *saci_2123* gene is up-regulated upon the exposure of cells to certain toxic compounds and a deletion mutant was found to be more vulnerable to a set of toxic compounds compared to the wild type strain. Moreover, overexpression of Saci_2123 increased the resistance of *S. acidocaldarius* to multiple toxic compounds and therefore this transport was designated as Smr1 (*Sulfolobus* multidrug resistance transporter 1).

**Chapter 4** presents a study on iron uptake in *S. acidocaldarius*. The genome of *S. acidocaldarius* specifies three putative iron substrate-binding proteins. The expression of these genes is up-regulated under conditions of iron deprivation, while their deletion affected growth under iron-limiting and rich conditions which could be attributed to a defect in the uptake of iron. These data suggest that iron uptake in *S. acidocaldarius* is binding protein dependent.

**Chapter 5** represent the first step in understanding the networks that underlying the regulation of cellular motility in Crenarchaeota. Two proteins, the FHA domain-containing protein ArnA and the vWA domain-containing protein ArnB that are involved in regulation of archaella expression, were identified in *S. acidocaldarius*. These proteins were in an earlier study found to be associated with the cell envelope derived membrane vesicles. Both proteins are phosphorylated by protein kinases *in vitro* and interact strongly *in vivo*. Phenotypic analyses revealed that these two proteins are repressors of archaella expression.

The thesis is concluded with a summary and outlook.

# 2

# DELETION OF CDVB PARALOGOUS GENES OF *SULFOLOBUS ACIDOCALDARIUS* IMPAIRS CELL DIVISION

Nuan Yang and Arnold J. M. Driessen

*Extremophiles* (2014) 18:331-339

## ABSTRACT

The majority of Crenarchaeota utilize the cell division system (Cdv) to divide. This system consists of three highly conserved genes, *cdvA*, *cdvB* and *cdv*C that are organized in an operon. CdvC is homologous to the AAA-type ATPase Vps4, involved in multivesicular body biogenesis in eukaryotes. CdvA is a unique archaeal protein that interacts with the membrane, while CdvB is homologous to the eukaryal Vps24 and forms helical filaments. Most Crenarcheota contain additional CdvB paralogs. In *Sulfolobus acidocaldarius* these are termed CdvB1-3. We have used a gene inactivation approach to determine the impact of these additional *cdvB* genes on cell division. Independent deletion mutants of these genes were analyzed for growth and protein localization. One of the deletion strains (*ΔcdvB3*) showed a severe growth defect on plates and delayed growth on liquid medium. It showed the formation of enlarged cells and a defect in DNA segregation. Since these defects are accompanied with an aberrant localization of CdvA and CdvB, we conclude that CdvB3 fulfills an important accessory role in cell division.

# INTRODUCTION

A major unresolved question is how archaea divide. Remarkably, the different phyla of archaea seem to employ at least three unrelated main mechanisms for cell division. Euryarchaeota and Nanoarchaeota presumably rely on a FtsZ-based mechanism, a protein that shows structural similarities to eukaryotic tubulin (Wang and Lutkenhaus, 1996; Lowe and Amos, 1998; Poplawski *et al.,* 2000; Makarova and Koonin, 2010), and possibly functionally resembles the bacterial cell division system (Cdv). In some Thermoproteales, an actin-like system appears to be in place (Makarova *et al.,* 2010; Ettema *et al.,* 2011), whereas the majority of Crenarchaeota and all Thaumarchaeota utilize the Cdv (Lindas *et al.,* 2008; Samson *et al.,* 2008). The Cdv system consists of three genes, *cdvA*, *cdvB* and *cdvC* that are widely conserved among Crenarchaeota. Importantly, this system shows homology to the eukaryotic ''endosomal sorting complex required for transport'' ESCRT machinery (Obita *et al.,* 2007; Hobel *et al.,* 2008; Carlton 2010). CdvC is homologous to the AAA-type ATPase, Vps4 (Obita *et al.,* 2007; Hobel *et al.,* 2008; Lindas *et al.,* 2008; Samson *et al.,* 2008), which is involved in multivesicular body biogenesis in eukaryotes (Hurley 2008; Henne *et al.,* 2011). Vps4 facilitates the recycling of the ESCRT-III complex (Babst *et al.,* 1998; Stuchell-Brereton *et al.,* 2007; Li and Blissard, 2012). CdvB is homologous to Vps24 which presumably functions as a core component of the ESCRT-III system and that can form helical filaments. Finally, CdvA is an α-helical peripheral membrane protein and thus far no eukaryotic homolog has been identified (Lindas *et al.,* 2008; Makarova *et al.,* 2010).

In Eukarya, the ESCRT machinery plays a pivotal role in a wide variety of membrane-linked highly conserved processes such as cytokinesis (Carlton and Martin-Serrano, 2007; Morita *et al.,* 2007, 2010; Raiborg and Stenmark, 2009), vesicles formation (Saksena *et al.,* 2007; Williams and Urbe, 2007; Hurley and Hanson, 2010) and budding of enveloped viruses (Morita and Sundquist, 2004; Carlton and Martin-Serrano, 2007; Peel *et al.,* 2011). The presence of the Vps4/ESCRT-III and Cdv system in eukaryotes and archaea (Makarova *et al.,* 2010), respectively, together with the proposed function in cytokinesis hints at an ancient conserved function (Lindas and Bernander, 2013). In this respect, the archaeal Cdv system also appears to be involved in multiple other membrane-linked processes next to cell division, such as membrane vesicle formation (Ellen *et al.,* 2009; Makarova *et al.,* 2010) and virus budding (Maaty *et al.,* 2006; Ortmann *et al.,* 2008; Wollert *et al.,* 2009; Snyder *et al.,* 2013). Recent electron cryotomography of ESCRT assemblies in dividing *Sulfolobus* cells suggests that spiraling filaments are involved in membrane scission (Dobro *et al.,* 2013).

An interaction network among Cdv proteins has been proposed for *Sulfolobales* based on a yeast two-hybrid study and biochemical verification. Similar to the eukaryotic system in which Vps4 and ESCRT-III are interacting partners (Kieffer *et al.,* 2008), CdvB interacts with CdvC (Obita *et al.,* 2007; Samson *et al*., 2008), and with CdvA (Moriscot *et al*., 2011; Samson *et al*., 2011). The binding between these interacting partners is not mutually exclusive, but there is no direct evidence that CdvA and CdvC interact. In analogy to the ESCRT-III system of eukaryotes, there are at least two extra CdvB paralogs with a possible relation to cell division in Crenarchaea. *Sulfolobales* contains three additional CdvB paralogs (Fig. 1) that are differentially expressed under conditions of cell cycle, viral infection and others. The *cdvB*1–3 genes do not localize in the vicinity to the *cdvABC* genes, but are positioned elsewhere on the chromosome. The CdvB proteins share a similar core of homology corresponding to the α-helix rich core ESCRT-III fold but they differ in the length while the CdvB1–3 paralogs miss the C-terminal tail of CdvB that has been implicated

in CdvA binding (Samson *et al*., 2008). The CdvB1–3 proteins interact with themselves and each other but unlike CdvB they do not interact with CdvC (Samson *et al*., 2008). Two of CdvB paralogs (CdvB1 and CdvB2, respectively) appear to share a common origin (Ettema and Bernander, 2009) and both were found to be present in secretory vesicles in *S. acidocaldarius* (Ellen *et al*., 2009) suggesting a role in vesicle formation. In addition, the *Sulfolobales* CdvB paralogs have been implicated in virus release (Maaty *et al*., 2006; Ettema and Bernander, 2009; Snyder *et al*., 2013). Importantly, the mechanistic implications of these associations and the respective roles of these CdvB paralogs still remain to be determined.

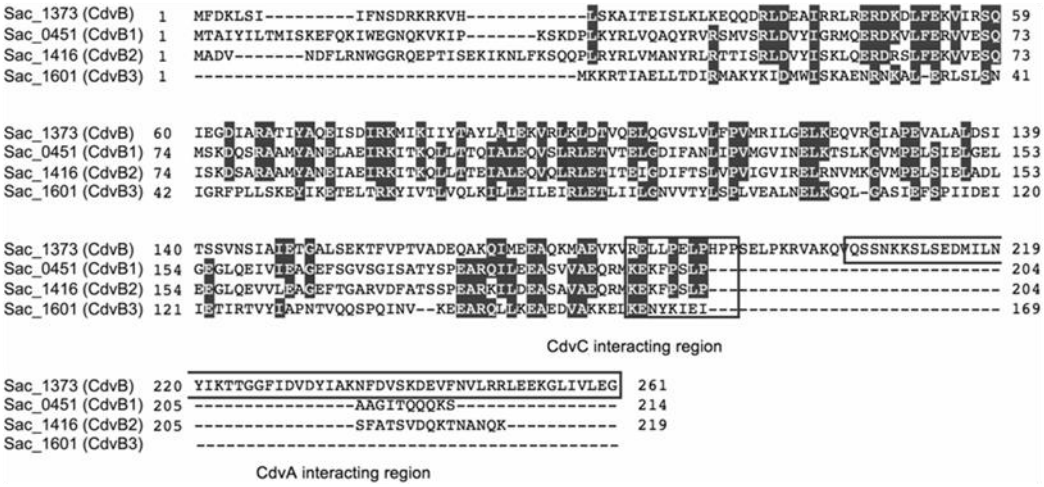


**Fig. 1** Multiple amino acid sequence alignment of CdvB and the CdvB paralogous proteins from *S. acidocaldarius*. Black indicates 100% identity between at least 3 of the 4 aligned sequences. The predicted CdvC and CdvA binding sites are indicated by the boxes.

In order to interrogate the role of the *cdvB* genes, we have used a genetic approach to construct and characterize deletion mutants of *cdvB* (*saci_1373*), *cdvB1* (*saci_0451*), *cdvB2* (*saci_1416*) and *cdvB3* (*saci_1601*). Except for the *cdvB* gene inactivation strain, all other deletion strains could be obtained although the resultant strains varied in the extent of their growth defect. Further analysis of the cell morphology and cellular localization of the various Cdv proteins indicate that the paralogous CdvB proteins function in cell division within particularly a more stringent role of CdvB3.

## MATERIALS AND METHODS

### Strains and growth conditions

*Sulfolobus acidocaldarius* MR31 (Reilly and Grogan, 2001) and all marker-less deletion mutants were grown aerobically at 78°C in Brock's medium (Brock *et al*., 1972) adjusted to pH 3.5 with sulfuric acid, supplemented with 0.2 % (wt/vol) tryptone, 20 µg/ml uracil, and when necessary 0.2 % (wt/vol) N-Z-Amine, 0.2 % (wt/vol) xylose, 50 mg/ml 5-fluoroorotic acid (5-FOA) and 0.64 % Gelrite (GmbH).

### Construction of the deletion mutants

To inactivate the target genes named *cdvB* (*saci_1373*), *cdvB1* (*saci_0451*), *cdvB2* (*saci_1416)* and

*cdvB3* (*saci_1601*), the upstream- and downstream-flanking regions of the gene of interest were cloned with the primer sets indicated in Table S1 and fused into the gene targeting vector pΔ2pyrEF (Wagner *et al*., 2009). The resulting plasmids were transformed into *S. acidocaldarius* MR31 and the individual *cdvB* deletion mutants were isolated as described previously (Ellen *et al*., 2010). Deletion mutants were validated by PCR.

**Growth curves and flow cytometry**

Pre-cultures of the wild-type and mutant strains were spread evenly onto the surface of Brock's plates, and incubated for 6 days at 78°C. Individual colonies were grown in liquid culture until the early log phase, diluted 50-fold into fresh medium. Growth of cells was continued and monitored by measuring the optical density at 600 nm. To determine cell size and DNA content, samples (0.2 ml) were taken at various time points, processed and subjected to flow cytometry with WinMDI 2.9 software as described (Gotz *et al*., 2007). Parameters that were measured are forward scatter to indicate cell size and DAPI fluorescence to DNA content. Cells were fixed in cold 70% ethanol, washed and re-suspended in 10 mM Tris-HCl pH 7.4 and 10 mM $MgCl_2$ at 4°C, then stained by DAPI for FACS analysis to determine the cells' size. Flow cytometry was performed with a FACSCanto flow cytometer (BD Biosciences).

**RNA isolation and transcriptional analyses**

Gene expression was quantified by real-time quantitative PCR (qPCR) using the primers indicated in Table S2 as described previously (Ellen *et al*., 2011). All primers were designed so that fragments of 290 bp were synthesized. Total RNA of the indicated strains at various time points was isolated with TRIzol (Invitrogen), additional DNase treatment using the Turbo DNA-free kit (Ambion) and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). The concentration of total RNA was measured with NanoDrop ND-1000. The gene expression levels were analyzed in three replicate samples with a MiniOpticon system (Bio-Rad) using the Bio-Rad CFX manager software.

**Immunostaining and fluorescence microscopy**

Samples from *S. acidocaldarius* cultures were fixed in cold ethanol, washed with phosphate-buffered saline with Tween20 (PBS containing 0.05% Tween20), incubated with primary and secondary antibodies for target proteins (Alexa Fluor 488 goat anti-rabbit IgG, or Alexa Fluor 488 goat anti-chicken IgG; Invitrogen), DAPI for DNA or FM4-64X for membrane staining (Invitrogen), respectively (Lindas *et al*., 2008; Samson *et al*., 2008). Protein fluorescence was imaged by microscopy using a Nikon Ti-E epifluorescence microscope (Nikon Instruments, Japan) equipped with a CFI Plan Achromat DM 1009 oil objective (Nikon Instruments), and a Hamamatsu ORCAFlash4.0 sCMOS camera (Hamamatsu Photonics, Hamamatsu City, Shizuoka Pref., Japan). Image analysis was done with the Nikon elements (Nikon Instruments) and Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA) software packages.

# RESULTS

**Deletion of the *cdvB* paralogs genes and growth phenotype**

To construct individual *cdvB* deletion mutants, vectors containing *pyrEF* flanked by the

upstream/downstream of the target gene were constructed and introduced into *S. acidocaldarius* MR31. Individual mutants were isolated for uracil auxotrophy and screened by PCR to verify the deletion of the respective *cdvB* genes (Fig. 2). This method failed to yield a deletion mutant of the *cdvB* gene (data not shown), but for each of the paralogous genes, *cdvB1–3*, individual mutants were obtained. These deletion strains differed from the wild-type and yielded small colonies on selective plates that varied in size depending on the mutant strain (Fig. 3a). In particular the colonies of the *ΔcdvB3* strain were extremely small but still visible. These data indicate that none of the *cdvB* paralogous genes is essential for growth of *S. acidocaldarius* but that the small colony size associated with the deletion strains signifies a growth defect.

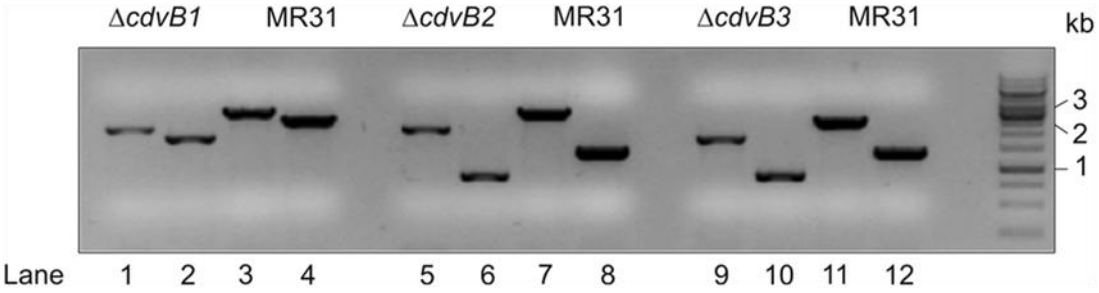


**Fig. 2** Verification of the gene inactivation of the paralogous *cdvB* genes. PCR analysis of individual strains for the absence of the *cdvB1* (*saci_0451*) gene (lane 1, 2), *cdvB2* (*saci_1416*) gene (lane 5, 6), *cdvB3* (*saci_1601*) gene (lane 9, 10), and presence of the *cdvB1* gene (lane 3, 4), *cdvB2* gene (lane 7, 8), *cdvB3* gene (lane 11, 12), in the genomic DNA derived from the wild-type and *ΔcdvB* deletion strains of S. acidocaldarius for the loss of the *cdv* gene, respectively. The PCR products were subjected to electrophoresis in 1 % agarose.

The growth phenotype of the wild-type and mutant strains was further examined at 78°C on Brock's liquid medium. Mutant strains grew much slower as compared to the wild-type (Fig. 3b), and again, the *ΔcdvB3* mutant strain showed the most severe effect with a prolonged lag phase but eventually cells started to grow. This was, however, a stable phenotype as upon re-inoculation of liquid growth medium with the various deletion strains, growth was again preceded by a long lag phase followed by near to normal growth (data not shown). These data demonstrate that in particular the *ΔcdvB3* mutant has a strong growth defect consistent with a possible role in cell division.

**Expression levels of *cdv* genes**

To examine the possible interrelationship of the expression of the *cdv* genes, quantitative PCR analysis was carried out on RNA isolated from the wild-type and individual *cdvB1–3* gene inactivation mutants harvested at different stages of growth. In the early log phase, the absence of *cdvB3* resulted also in a reduction in the transcriptional levels *cdvA*, *cdvB* and *cdvC* (Fig. 4a), whereas in the *ΔcdvB1* and *ΔcdvB2* strains, expression levels were rather similar as with the wild-type strain (data not shown). In mid log phase, the expression of *cdvA*, *cdvB* and *cdvC* in the *ΔcdvB3* strain was indistinguishable from the wild-type levels (Fig. 4b), consistent with the growth phenotype of these cells.

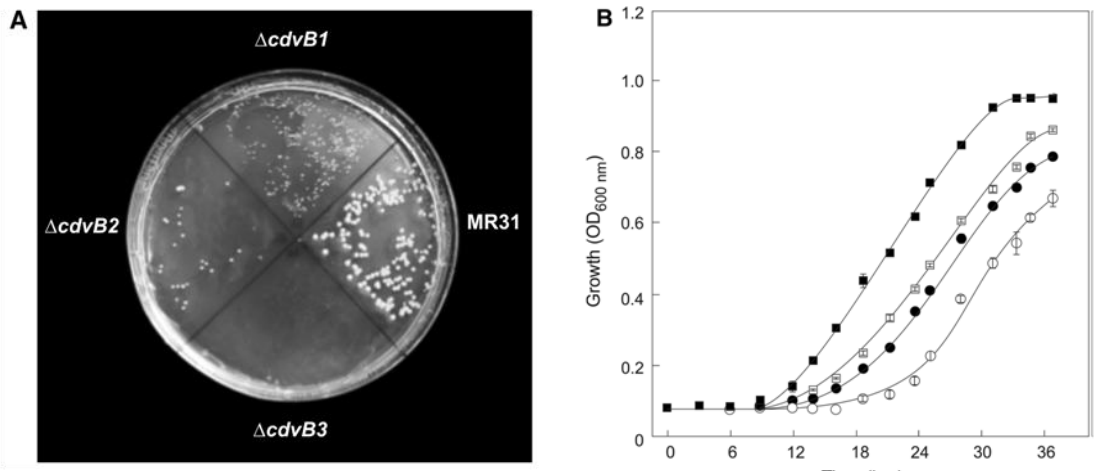


**Fig. 3 a** Colony formation by *S. acidocaldarius* MR31 and the derived *cdvB* gene inactivation strains after incubation for 8 days on plates. **b** Growth of the wild-type and *ΔcdvB* paralogs strains in liquid culture. Symbols represent: filled squares wild-type, open squares *ΔcdvB1* (*saci_0451*), filled circles *ΔcdvB2* (*saci_1416*) and, open circles *ΔcdvB3* (*saci_1601*). Each value in the curves is the mean of three independent replicates.

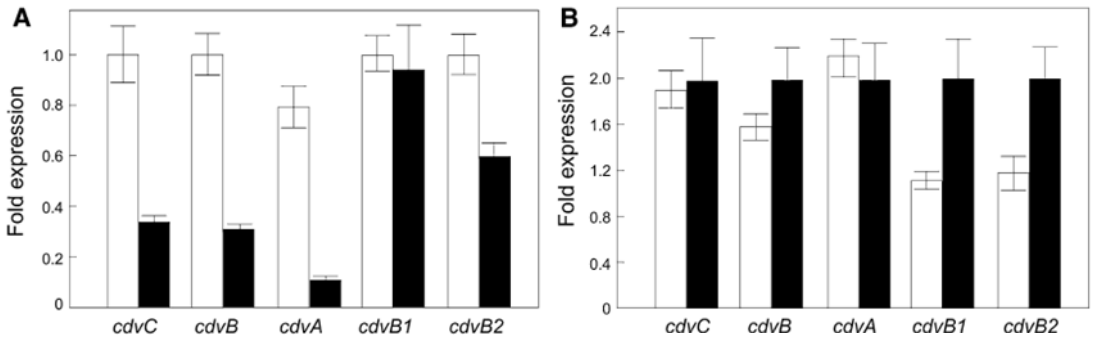


**Fig. 4** Expression levels of the *cdv* genes from the wild-type (white) and the *ΔcdvB3* (black) strains during the **a** early and **b** mid log phase. Expression was determined by real-time quantitative PCR using the primers indicated in Table S1 and normalized relative to the expression of the *cdv* genes in the early and mid log phase using the *secY* gene as a reference. Early and mid log phases correspond to $OD_{600}$ of 0.2 and 0.5, respectively. Data are presented as mean ± SD, n = 3

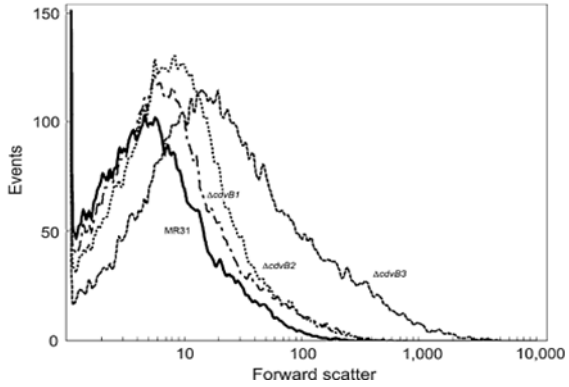


**Fig. 5** FACS analysis of the cell size distribution of cells collected from the early log phase. The X-axis indicates relative cell size. Cell size distribution curve of the wild-type, *ΔcdvB1*, *ΔcdvB2*, *ΔcdvB3*, as indicated by the solid line, dashed line, dotted-dashed line and dashed line, respectively. Further details are as in the ''Materials and methods''

**Cell size and flow cytometry**

Generally, *S. acidocaldarius* cells are roughly coccoidal shaped, and rather similar in size typically in the range of one micrometer. Cells of the wild-type and mutants strains were collected from the various growth stages, labeled with DAPI to stain the DNA, and subjected to flow cytometry and fluorescence microscopy to examine their size and DNA content. As evidenced by flow cytometry (Fig. 5) and fluorescence microscopy (Fig. 6) a substantial proportion of the *cdvB1–3* deletion cells showed a dramatic phenotype with the appearance of considerably enlarged cells, up to two/three times the diameter of wild-type cells. These cells also show an intense DAPI staining indicative of an elevated DNA content. With growth, the number of enlarged cells declined (data not shown). Again, the *ΔcdvB3* mutant strain showed the strongest phenotype. Previously, it has been reported that expression of an allele of *cdvC* resulted in the formation of large cells with a highly elevated content of DNA (Samson *et al*., 2008) and this was attributed to a cell division defect. The presence of the enlarged cells with increased DNA content in the individual *cdvB* gene inactivation strains therefore suggests a defect in cell division.

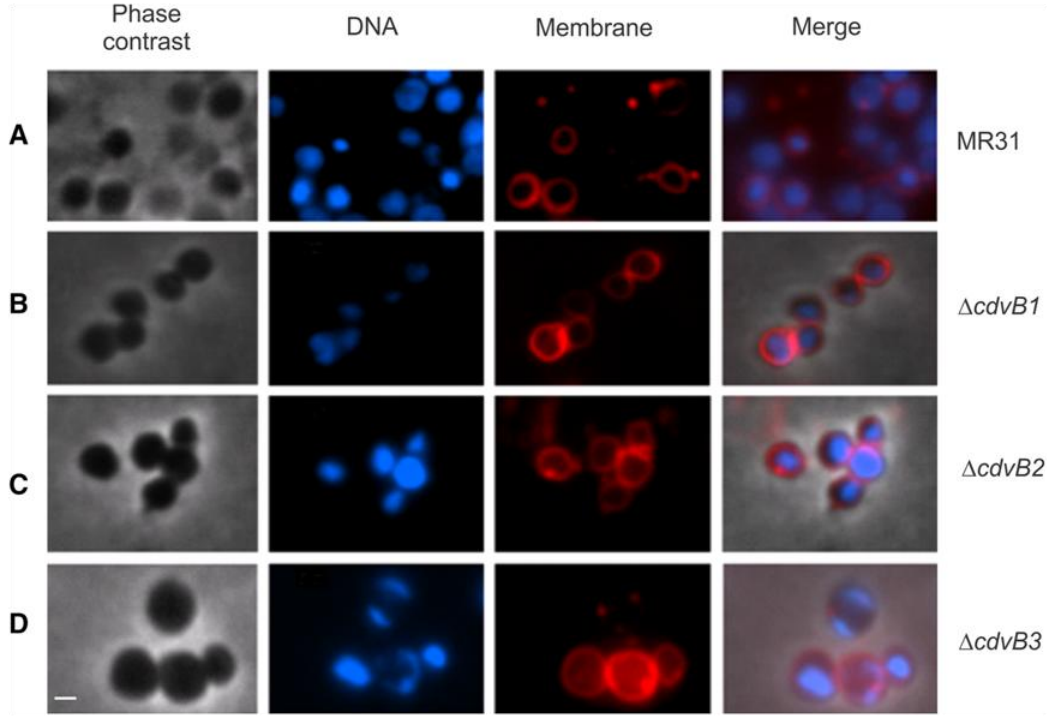


**Fig. 6** Fluorescence microscopic analyses of early log phase grown cells of the *S. acidocaldarius* wild-type and *ΔcdvB* paralogs strains. Panels show the phase contrast, DAPI staining for DNA (blue), FM4-64 staining for membrane (red), and merged images. Scale bar 1µm. Cells were grown in Brock's medium.

**Cellular localization of Cdv proteins**

Cells from liquid medium-grown wild-type and mutant strains were harvested at various stages of growth, fixed in ethanol and immunostained with specific antibodies directed against CdvA, CdvB, CdvB3 and CdvC, and subsequently examined by fluorescence microscopy. In early log phase wild-type cells, CdvB typically assembles into a band structure (Fig. 7a–e) at mid-cell between the segregated or non-segregated nucleoids, deconvolution revealed that the band of CdvB forms a ring-like structure (Lindas *et al*., 2008; Samson *et al*., 2008), where eventually the ring

structure constricts to separate the daughter cells. CdvA displayed a similar localization pattern and structure as CdvB (Fig. 8a–d). Bands or rings of CdvA and CdvB were also observed in some cells where the nucleoids were not yet segregated, suggesting that formation of the band or ring-like structure precedes detectable membrane ingression even before nucleoid segregation

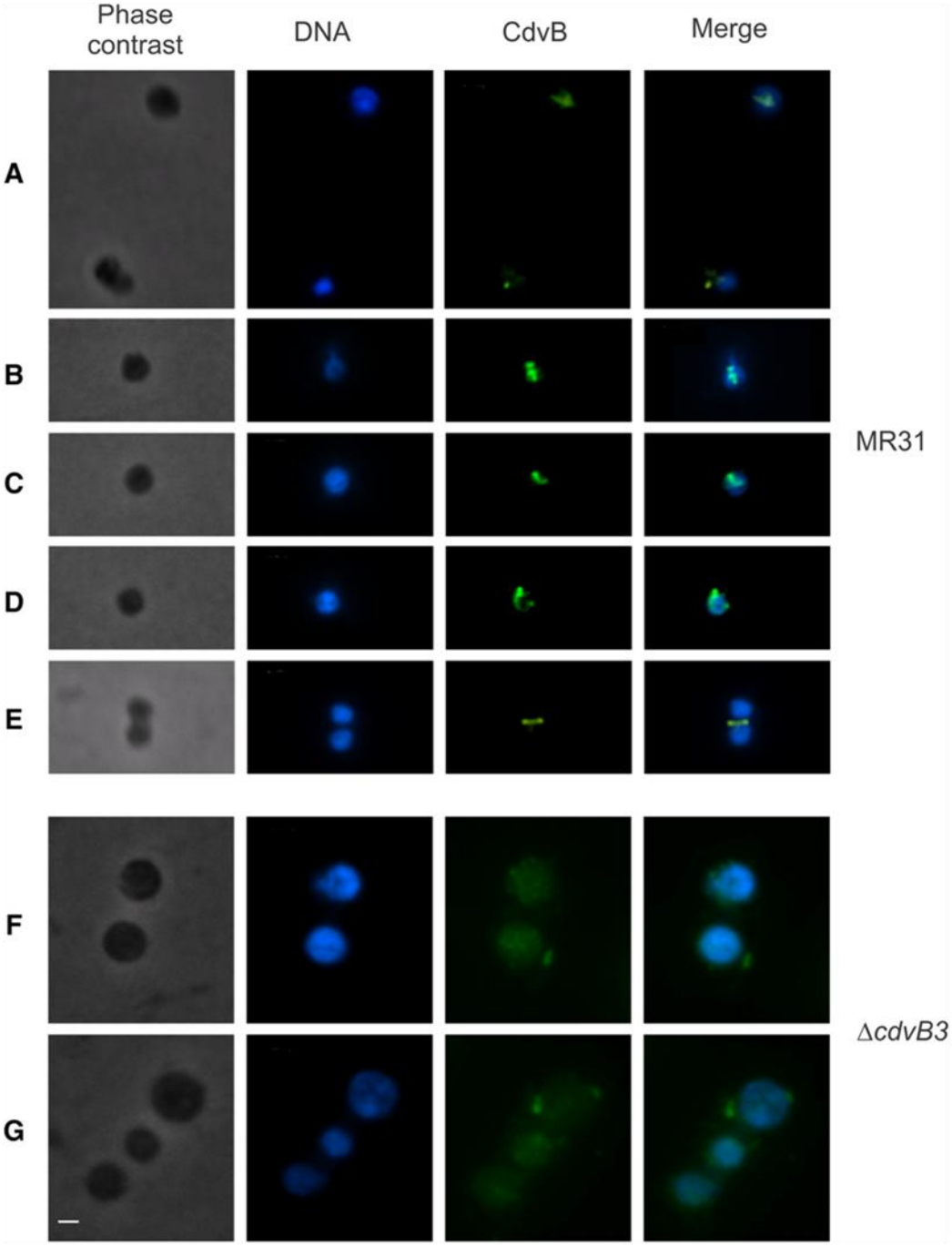


**Fig. 7** Cellular localization of CdvB in early log phase grown *S. acidocaldarius* wild-type and *ΔcdvB3* cells. Panels show the phase contrast, DAPI staining for DNA (blue), immunolocalization with anti-CdvB antibodies (green) and merged images. The scale bar corresponds to 1 µm. **a–e** CdvB structure in the wild-type strain shows a band or belt (ring-like structure) of CdvB traversing the middle of the cell, yet nucleoids have not segregated. **e** The separation of the nucleotides as the membranes constrict during division. **f, g** CdvB structure in the *ΔcdvB3* mutant strain.

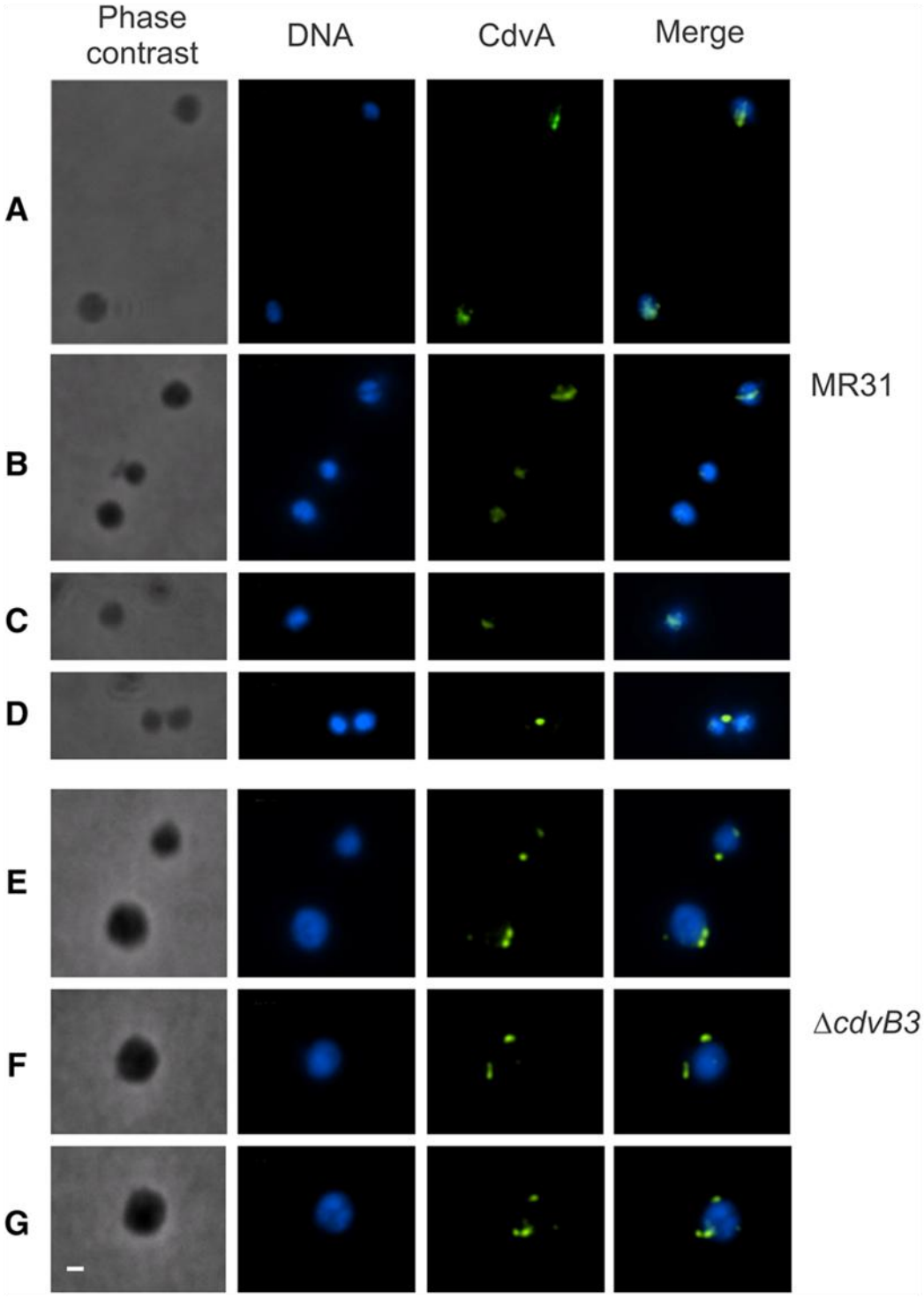


**Fig. 8** Cellular localization of CdvA in early log phase grown *S. acidocaldarius* wild-type and *ΔcdvB3* cells. Panels show the phase contrast, DAPI staining for DNA (blue), immunolocalization with anti-CdvA antibodies (green) and merged images. The scale bar corresponds to 1 µm. **a–d** The CdvA structure in wild-type strain. **a–c** A band or belt of CdvA traversing the middle of the cell, yet nucleoids have not segregated. **d** The constriction of the nucleotides as membranes constrict during division. **e–g** The CdvA structure in the *ΔcdvB3* mutant strain.

(Samson *et al*., 2008). CdvC also formed ring-like structures, while with the CdvB3 specific antibodies no specific structures could be observed (data not shown). The cellular localization of the Cdv proteins was also examined in the individual *ΔcdvB* strains. The pattern of localization of CdvB and CdvA in the *ΔcdvB1* and *ΔcdvB2* strains was very similar as compared to the wild-type (data not shown). However, only two or three spots of CdvA could be discerned in the enlarged cells of *ΔcdvB3* strain (Fig. 8e–g), while the structure of CdvB was hard to assess because of the low CdvB fluorescence signal (Fig. 7f, g). There were no distinguishable bands or ring-like structures in *ΔcdvB3* mutant cells and also membrane invagination does not seem to occur in the enlarged cells as evidenced by the membrane stain (Fig. 6). In a substantial fraction of the enlarged cells, two separated nucleoids could be distinguished at opposite poles of the cell with no indication of invagination in the middle. These results demonstrate an aberrant cellular localization of CdvA in the *ΔcdvB3* mutant cells further implying a role of CdvB3 in cell division.

Because of the loss of CdvB3, cells with duplicated genomes may not be capable of dividing on time, and continue to grow in size in early log phase. Nevertheless, it is possible that these enlarged cells stop growing and/or eventually die, while the subpopulation of smaller cells manages to divide at near-normal size as even the *ΔcdvB3* mutant continues to grow even though delayed. This is accompanied with a normal wild-type expression of the *cdvABC* genes that are lower expressed in the *ΔcdvB3* strain during early log phase. Although the lower expression of these genes may be responsible for the reduced growth rate which is in particular strong with plate-grown cells, the restoration of expression during mid log phase in liquid culture suggests an important, yet nonessential function of CdvB3 in the actual division process. Interestingly, only the expression of *cdvB3* is cell cycle related (Ettema and Bernander, 2009) further suggesting a role in cell division. In the wild-type strain, cell division seems to be associated with a mid-cell localization of a ring-like structure of the CdvA protein that may be bound to the membrane and possibly also interacts with the DNA. This membrane bound ring then recruits CdvB to form similar ring-like structure. As the ring constricts, possibly at the expense of ATP hydrolysis by CdvC, the cell divides into the two daughter cells. A prerequisite to nucleoid segregation and recruitment of CdvB is the assembly of CdvA. In this regard, due to the absence of CdvB3 in the mutant strain, the CdvA protein seems to assemble in a spot structure rather than a band or ring-like structure. Likely, this structure is defective in the recruitment of CdvB, hence the low CdvB fluorescence in these cells. This process might be necessary to initiate cell constriction and division. Our data suggest that CdvB3 fulfills a coordination function between the Cdv proteins during cell division.

## ACKNOWLEDGMENTS

Thanks to Dirk-Jan Scheffers for help with the fluorescence microscopy, and Maarten Mols for assistance in the flow cytometry. We are grateful to Ralf Bernander (Stockholm University, Sweden) for kindly providing the Cdv antibodies.

# 3

# THE *SACI_2123* GENE OF THE HYPERTHERMOACIDOPHILE *SULFOLOBUS ACIDOCALDARIUS* ENCODES AN ATP-BINDING CASSETTE MULTIDRUG TRANSPORTER

Nuan Yang and Arnold J. M. Driessen

*Extremophiles* (2015) 19:101-108

## ABSTRACT

Multidrug resistance (MDR) transporters are capable of secreting structurally and functionally unrelated toxic compounds from the cell. Among this group are ATP-binding cassette (ABC) transporters. These membrane proteins are typically arranged as either hetero- or homo-dimers of ABC half-transporters with each subunit consisting of a membrane domain fused at the C-terminus to an ATP-binding domain, or as full transporters in which the two subunits are fused into a single polypeptide. The *saci_2123* gene of the thermoacidophilic archaeon *Sulfolobus acidocaldarius* is the only gene in the genome that encodes an ATP-binding cassette half-transporter, while a homologous gene is present in the genomes of *S. solfataricus*, *S. tokodaii* and *S. islandicus*. Saci_2123 shares homology with well-characterized bacterial and mammalian MDR transporters. The *saci_2123* gene is up-regulated when cells are exposed to drugs. A deletion mutant of *saci_2123* was found to be more vulnerable to a set of toxic compounds, including detergents, antibiotics and uncouplers as compared to the wild-type strain, while the drug resistance could be restored through the plasmid-based expression of *saci_2123*. These data demonstrate that Saci_2132 is an archaeal ABC-MDR transporter and therefore it was termed Smr1 (*Sulfolobus* multidrug resistance transporter 1).

# INTRODUCTION

Microorganisms have developed diverse mechanisms to cope with toxic compounds in their environment. This includes mechanisms such as enzymatic inactivation or modification of the toxic compound, or alterations in the membrane composition in order to reduce the permeability of toxic compounds (Bush and Miller, 1998; Nikaido 2003; Ruiz 2003). A further mechanism is the active extrusion of toxic compounds from the cell by multidrug efflux transporters (Higgins 2007). These systems are equipped with a broad substrate specificity resulting in a phenomenon termed multidrug resistance (MDR) where cells become resistant to multiple drugs with unrelated structures. MDR emerges as a major resistance-conferring mechanism against antibiotics in pathogenic microorganisms. Therefore, the increasing incidence of MDR has a profound impact on the treatment of infectious diseases as well as the development of novel therapeutics. MDR may also confer resistance against xenobiotics and other adverse molecules in the environment. MDR is mediated by a dedicated groups of membrane transporters that fall into five families, i.e., the Major Facilitator Superfamily (MFS), the Resistance-Nodulation-Cell Division superfamily (RND), the Small Multidrug Resistance family (SMR), the Multidrug and Toxic compounds Extrusion family (MATE), and the ATP-binding cassette superfamily (ABC) (Zgurskaya and Nikaido, 2000; Lubelski *et al.,* 2007). All of these transporters are pleiotropic towards the substrates they recognize. Four of the families belong to the secondary transporters that use the proton motive force as energy source. On the other hand, the ABC-MDR transporters derive their energy from the binding and hydrolysis of ATP, which triggers conformational changes in the transporter driving the excretion of drugs into the external medium against considerable concentration gradients (Holland *et al.,* 2003). ABC transporters are a large number of ubiquitous and functionally versatile proteins and constitute one of the largest protein families widely distributed in all taxa from bacteria, archaea to humans (Holland *et al.,* 2003; Lubelski *et al.*, 2007). ABC importers that are involved in solute uptake are only found in bacteria and archaea and in general require an additional extracellular substrate binding protein (Lee *et al.,* 2007). In particular for sugar uptake, various ABC transporters have been functionally characterized in hyperthermo (acido-) philic archaea (Albers *et al.,* 2004). Also ABC-MDR exporters have been found in the genomes of all organisms sequenced to date. ABC exporters typically consist of two characteristic regions: a poorly conserved transmembrane domain (TMD) with 6 transmembrane segments, and a highly conserved nucleotide-binding domain (NBD) (Hollenstein *et al.,* 2007). Unlike with ABC importers, these two domains are fused into a single polypeptide in ABC exporters, and this architecture corresponds to so-called "half-transporters". The functional unit consists of either a homo- or heterodimer of two subunits. In eukaryotes, these subunits may also be fused into one large polypeptide termed "full-transporter" (Kerr 2002).

ABC-MDR transporters have been intensively investigated in mammals in relation to resistance of tumor cells to cytotoxic drugs and this includes the well-characterized MDR transporters P-glycoprotein, MRP1 and BCRP (Kerr 2002). In bacteria, MDR transporters provide resistance to antibiotics, xenobiotics and other adverse toxic compounds including organic solvents (Yen *et al.,* 2010). These transporters likely fulfill an important role in the cellular protection against natural occurring oranthropogenic toxic compounds (Roldán *et al.,* 2008). ABC protein mediated extrusion may also be of relevance for archaea as many thrive in extreme environments containing toxic chemicals (Albers *et al.,* 2004). For instance, archaea were found in oil-containing environments which are ecological niches contaminated by heavy metals,

heterocycles and other toxic compounds (Bini 2000). Robustness of archaea towards extreme environmental conditions has largely been attributed to their unique cell envelope and membrane harboring (membrane-spanning) ether lipids. However, little is known about the functional role of MDR transporter in archaea. So far only indirect evidence hints at the presence of an ABC-MDR transporter in *Haloferax volcanii* (Kaidoh *et al.,* 1996), but this system has not been functionally studied.

Here, we present the first identification and characterization of an ABC-multidrug protein from Archaea, specifically the crenarchaeote *Sulfolobus acidocaldarius*. *Sulfolobales* contain one gene encoding an ABC transporter with a domain organization typical for ABC-MDR transporters. A deletion mutant of this gene, i.e., *saci_2123* showed an increased drugs sensitivity, whereas the phenotype could be rescued by the plasmid based expression of *saci_2123*. These data indicate that Saci_2123 is a MDR-ABC transporter, demonstrating that this mechanism of protection against toxic compounds is conserved in all domains of life.

## MATERIALS AND METHODS

**Strains and cultivation**

The uracil auxotrophic parental strain *Sulfolobus sacidocaldarius* MR31 (Reilly and Grogan, 2001) and the derived marker-less *Δsaci_2123* strain were grown aerobically at 78°C in Brock's basal medium (Brock *et al.,* 1972) adjusted to pH 3-3.5 using sulfuric acid. For growth on plates, the medium was supplemented with 0.2 % (w/v) trypton, 20 μg/ml uracil, and when necessary 0.2% (w/v) N-Z-amine, 0.2% (w/v) xylose, 50 mg/ml 5-fluoroorotic acid (5-FOA). The growth of cells was monitored at 600 nm using a Novaspec™ Plus Visible Spectrophotometer (Amersham Biosciences).

**Strain construction**

To construct a deletion mutant strain of *saci_2123*, the up- and downstream of the gene (~1000 bp) were cloned with the primer sets indicated in Table S1 and fused into the gene targeting vector pΔ2pyrEF (Wagner *et al.,* 2009). The resulting methylated plasmid was transformed into parental strain *S. acidocaldarius* MR31. The deletion mutant was isolated and verified by PCR and DNA sequencing as described (Ellen *et al.,* 2010).

To complement the *S. acidocaldarius Δsaci_2123* strain, the fragment containing the *saci_2123* gene with its up- (~800 bp) and downstream (~500 bp) regions contain the native promoter and terminator sequences were amplified from *S. acidocaldarius* genomic DNA and cloned into the complementation vector pSVA1450 (Wagner and Albers, unpublished) using *E. coli* DH5α. The methylated plasmid was isolated and transformed into *S. acidocaldarius Δsaci_2123* as described (Wagner *et al.,* 2009).

**RNA isolation and Expression analysis**

The expression of *saci_2123* upon treatment of the cells with various toxic compounds was analyzed by real-time quantitative PCR (qPCR) as described (Ellen *et al.,* 2011). *S. acidocaldarius* MR31 was incubated in the presence or absence of sublethal concentrations of 2, 4-dinitrophenol (DPN) and Ethidium bromide (EtBr) for 2 or 4 hrs, where upon total RNA was isolated with the

TRIzol (Invitrogen) reagent, followed by additional DNase treatment. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). The concentration of purified total RNA was determined with a NanoDrop ND-1000. The gene expression was quantified in three replicate samples with a MiniOpticon system (Bio-Rad), the expression of the house-keeping gene *secY* was used as a control. The primers (Table S2) were designed such that fragments of 290 bp were synthesized.

**Drug susceptibility analyses**

Strains were grown in liquid Brock's culture until the early log phase, diluted 50-fold into fresh medium containing various concentrations of different toxic compounds as indicated in the results section. Growth of the cells was monitored in time, and the concentration of the toxic compounds causing inhibition of growth rate by 50% ($IC_{50}$ values) was determined in triplicate.

# RESULTS

**Bioinformatics analysis of Saci_2123**

The genome sequence of *S. acidocaldarius* was analyzed for the presence of putative ABC type multidrug transporters. The genome contains 28 genes encoding members of the ABC Superfamily. Nearly all of these systems have a domain organization typical for ABC type uptake systems with a solute binding protein (data not shown, Albers *et al.,* 2004). Secondary structure predicted using TMHMM version 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) indicates that there is only one gene, i.e., *saci_2123* which encodes a so-called 'half-transporter' consisting of a membrane domain with six predicted transmembrane segments fused at the C-terminus to an ATP binding domain (Fig. 1A). This organization is typically associated with multidrug resistance in bacteria and eukaryotes, and the functional transporter then consists of a dimeric assembly of the half-transporters. In eukaryotes, MDR transport can also be associated with 'full-transporters' where the two subunits are fused into a single polypeptide. However, no such protein is specified by the *S. acidocaldarius* genome. The *saci_2123* gene encodes an 861-amino acid long membrane protein with a total molecular mass of 92 kDa. Its amino acid sequence aligns with known ABC-MDR transporter such as the N- and C-terminal halves of the human MDR1 protein (P-glycoprotein), and the bacterial half-transporters LmrA from *Lactococcus lactis* and MsbA of *E. coli* (Fig. 1A). The highest level of sequence homology is confined to the C-terminal ATP binding domain, which contains all functional ABC sequence characteristics including the Walker A and B motifs, and the ABC signature motif. Overall, the structural organization of Saci_2123 is similar to that of bacterial ABC MDR half-transporters except that the protein is significantly longer than the bacterial systems because of the presence of a DUF1854 domain at the C-terminus (Fig. 1A). This is a functionally uncharacterized domain that may be involved in ligand binding and so far has only been found in uncharacterized proteins. Further database analysis indicates the presence of genes specifying full length homologs of Saci_2123 in *S. solfataricus* (Sso3012), *S. tokodaii* (ST1099) and *S. islandicus* (LS215_2517), and several other (hyper-) thermoacidophilic crenarcheota: *Caldivirga maquilingensis* (WP_012186138.1), *Thermoproteus uzoniensis* (WP_013679093.1), *Acidilobus saccharovarans* (WP_013266623.1), *Acidianus hospitalis* (WP_013775193.1). A full length homolog is also found in the *Nanoarchaeote* Nst1 (WP_004578004.1) that has been isolated from the Obsidian Pool in Yellowstone park living in symbiosis with a crenarchaeal host (Podar *et al.,* 2013).

Remarkably, a homologous gene is present in the bacterium *Cupriavidus basilensis* (WP_006162866.1), which is an important xenobiotics degrader (Cserháti *et al.,* 2012).

Also, in the genomes of the other *Sulfolobales*, there is only one candidate gene that encodes an ABC half-transporter. Interestingly, the *saci_2123* gene localizes in a genomic region that contains genes involved in sugar metabolism (Fig. 1B). However, this genomic context is not conserved in *S. solfataricus* and *S. tokodaii* consist with the notion that Saci_2123 encodes an ABC exporter rather than a binding protein dependent ABC transporter involved in sugar uptake.

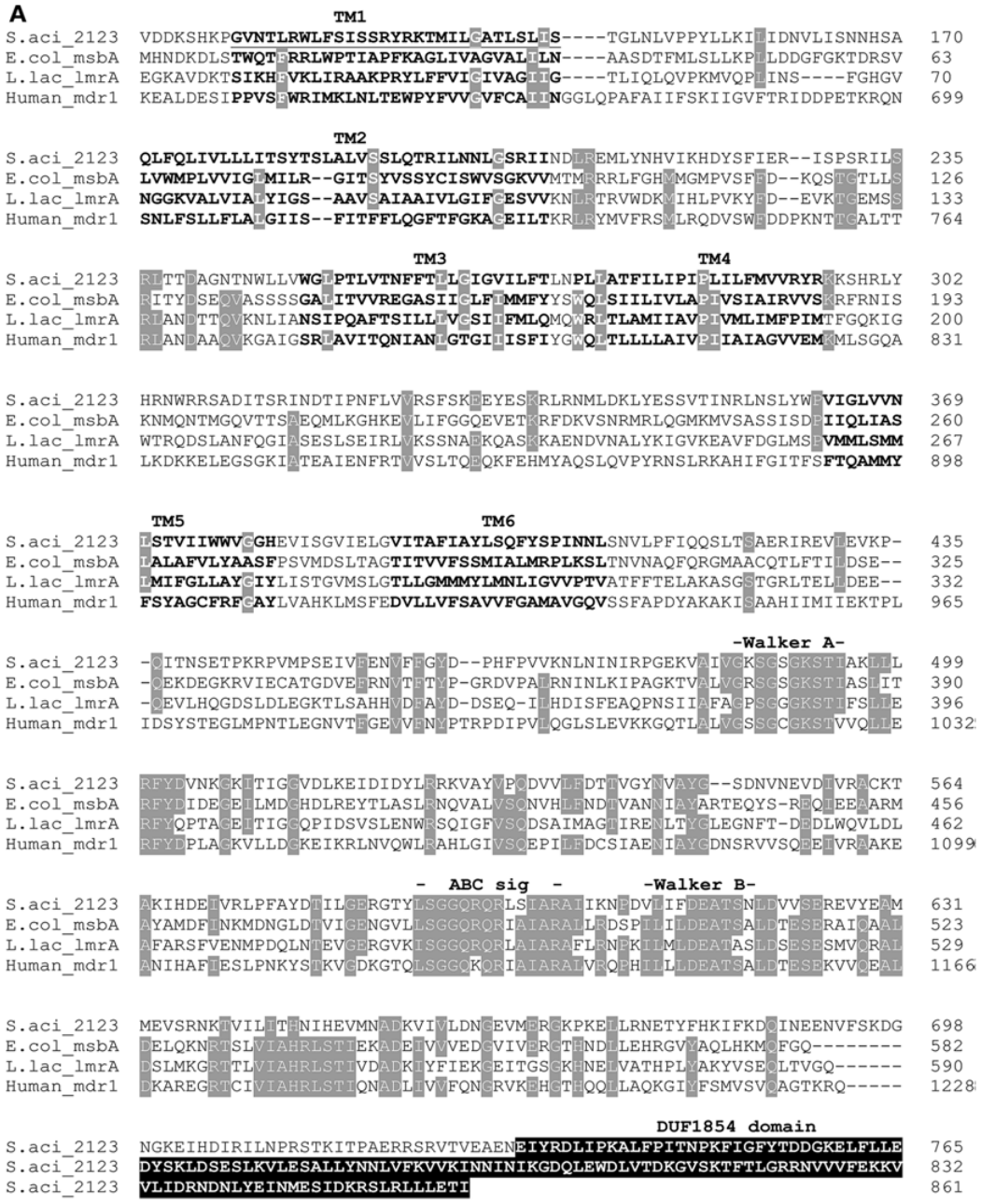


**Fig. 1** Sequence alignment of Saci_2123 with MDR transporters and genomic organization. **a** Multiple amino acid sequence alignment of Saci_2123 (Smr1) with the MDR transporters MsbA of *Escherichia coli*, LmrA of *Lactococcus lactis* and the C-terminal half of the human MDR1. Identical residues are shaded gray. Transmembrane regions are indicated in bold, and the Walker A and B, the ABC signature motif and the DUF1854 regions are shown. The alignment was done with ClustalW.

Fig. 1 continued

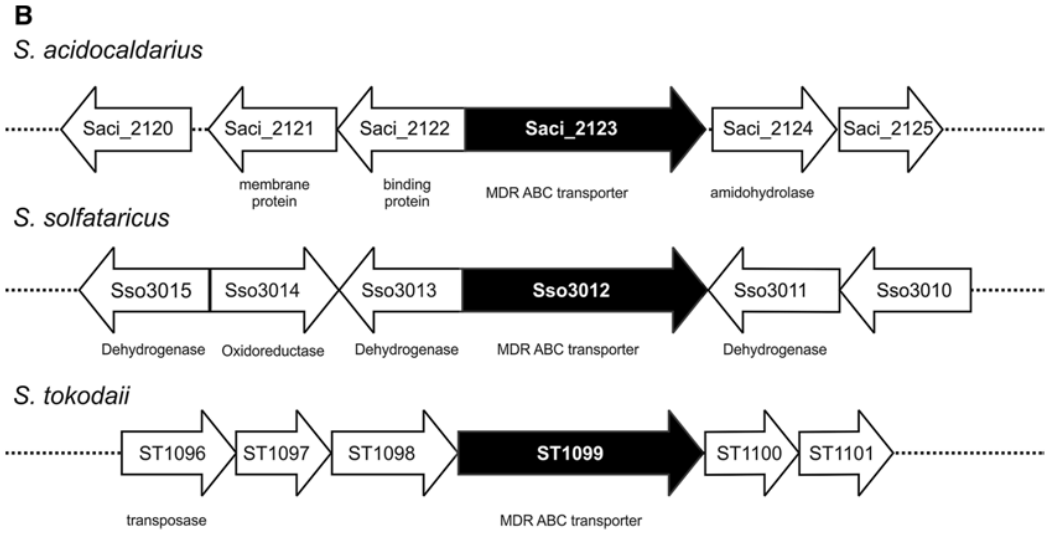


**Fig. 1** Sequence alignment of Saci_2123 with MDR transporters and genomic organization. **b** Genomic organization of the *smr1* gene of *Sulfolobus acidocaldarius*, *S. solfataricus* and *S. tokodaii*. The putative MDR ABC transporter genes are indicated in black, with the neighboring genes in white and when annotated, a possible function of the gene product is listed. The other genes specify hypothetical proteins.

### Deletion of the *saci_2123* gene and growth phenotype

To further analyze the physiological role of *saci_2123 in S. acidocaldarius,* its gene was deleted. Herein, a vector containing *pyrEF* flanked by the up and down-stream regions of *saci_2123* were constructed and introduced into the uracil auxotrophic background strain *S. acidocaldarius* MR31. The desired gene deletion mutant was generated by a double crossover recombination event and the successful deletion of *saci_2123* was confirmed by PCR in comparison with the MR31 reference strain (Fig. 2A) using the indicated primer sets (Table S1). The *saci_2123* deletion mutant showed no apparent defect in growth on Brock's mineral medium as compared to the wild type strain MR31 (Fig. 2B) suggesting that it does not specify a critical function under laboratory growth conditions.

To study the possible role in drug resistance, the *saci_2123* deletion mutant and parental strain MR31 were exposed to a range of drugs and the drug susceptibility of the cells was expressed in half-maximal growth inhibitory drug concentration ($IC_{50}$). Toxic agents that were tested were: antibiotics such as tetracycline, oxytetracycline, chloramphenicol and minocycline; detergents such as sodium dodecyl sulfate (SDS) and cholate; uncouplers, including carbonyl cyanide 3-chloro phenylhydrazone (CCCP), 2, 4-dinitrophenol (DNP); membrane permeable compounds such as tetraphenylphosphonium ion ($TPP^+$); heterocyclic dyes such as rhodamine 6G and acridine orange; DNA intercalating dyes such as ethidum bromide; and several other chemicals. As expected, the toxicity of these compounds varied over a wide concentration range (Table 1). However, the *Δsaci_2123* strain was found to be up to 2-fold more sensitive to the uncouplers CCCP and DNP, SDS and the antibiotics tetracycline and chloramphenicol (Table 1) to which the parental strain showed a higher tolerance. With a number of compounds no difference in susceptibility was observed (Table 1 and Fig. 3C). To determine if the observed drug resistance is indeed due to the expression of *saci_2123* and not associated to a polar effect on the expression

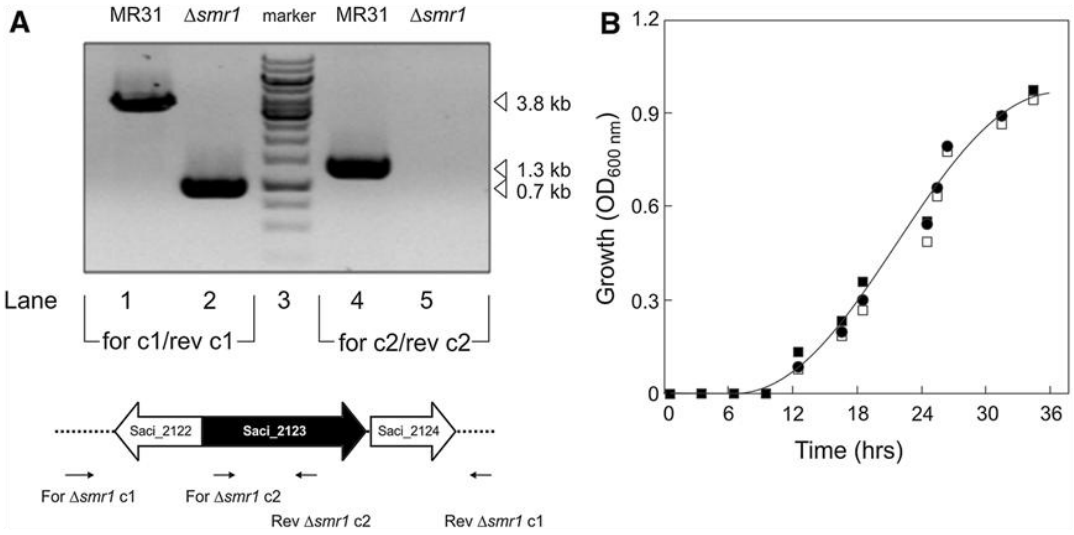


**Fig. 2** Verification of the *smr1* gene deletion and growth. **a** PCR analysis was carried out with the genomic DNA derived from the wild type and *Δsaci_2123* (*Δsmr1*) mutant strain of *S. acidocaldarius* using the indicated primers targeting the complete or an internal part of the *smr1* gene. The positions of the primer sets used to confirm the deletion of the *smr1* gene are indicated. **b** Growth of *S. acidocaldarius Δsmr1* with plasmid pSVA1450 (filled circle) or pSVA*smr1* (unfilled square) and the parental MR31 strain (filled square). Each value in the curves is the mean of three independent replicates.

of neighboring genes, the *saci_2123* gene was cloned under control of its own promoter into the expression vector pSVA1450 and transformed into the *Δsaci_2123* strain. The introduction of the plasmid completely restored the susceptibility of the deletion strain to CCCP and DNP to levels comparable or even slightly higher than the parental strain (Fig. 3), whereas the empty plasmid was without an effect. Taken together, these data suggest that Saci_2123 is a MDR transporter, and thus the gene was termed *smr*1 (*Sulfolobus* multidrug resistance transporter 1).

**Transcriptional up-regulation following drug exposure**

In bacteria, the innate defense mechanism conferred by MDR genes is often observed as an alteration of the expression level in response to drug exposure (Grkovic *et al.*, 2002). This allows cells to respond to the presence of toxic compounds in the environment, and increase the capacity to excrete these chemicals to relieve the cells from the environmental stress. To determine if the expression of *smr1* also responds to the presence of toxic compounds, the relative gene expression was quantified by qPCR, following exposure to sub-inhibitory concentrations of DNP and ethidum bromide. The latter appears not to be a substrate for Smr1 even though significant toxicity is exerted onto the cells. While, the transcription of *smr1* increased after treatment with DNP, exposure to ethidum bromide did not elicit such response (Fig. 4).

## DISCUSSION

Although the role of ABC-MDR proteins has been studied in great detail in antibiotics and xenobiotics resistance in bacteria and in the resistance against cytotoxic agents in mammals and other eukaryotes, much less is known about MDR transporters in archaea despite the fact that

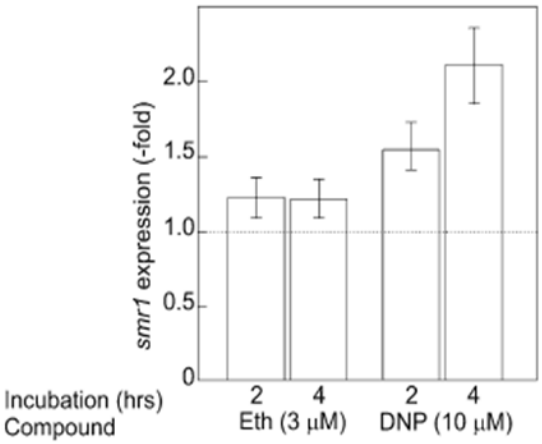


**Fig. 4** Expression of the *smr1* gene in *S. acidocaldarius* upon the exposure to sublethal concentrations of toxic chemicals. Expression of *smr1* in *S. acidocaldarius* M31 was determined by real-time quantitative PCR using the primers indicated in Table S2, normalized relative to the *secY* gene which was used as a reference, and expressed as fold-expression relative to the *smr1* expression in cells not exposed to a toxic drug. DNP and ethidium bromide (Eth) were used at a concentration of 3 and 10 μM, respectively. Incubation was for 2 and 4 h. The standard error of the mean for 3 independent experiments is shown.

**Table 1** Susceptibility of *S. acidocaldarius* MR31 and *Δsmr1* strain for toxic compounds

| Drug | $IC_{50}$ (μM)[a] | | Relative resistance[b] |
|---|---|---|---|
| | Wild type | Δ*smr1* | |
| SDS | 72.6 ± 5.5 | 43 ± 4.0 | 1.7 |
| DNP | 20 ± 2 | 15 ± 2 | 1.3 |
| CCCP | 9.5 ± 0.5 | 6.4 ± 0.5 | 1.5 |
| Fusidic acid | 4.9 ± 0.5 | 3.8 ± 0.4 | 1.3 |
| Minocycline | 3.5 ± 0.3 | 2.3 ± 0.4 | 1.5 |
| Tetracycline | 9.1 ± 0.7 | 4.9 ± 0.4 | 1.9 |
| Oxytetracycline | 12.9 ± 0.9 | 8.4 ± 0.7 | 1.5 |
| Chloramphenicol | 6.5 ± 0.5 | 3.2 ± 0.5 | 2.0 |
| Tetraphenylphosphonium ion | 18.5 ± 0.7 | 18.5 ± 0.7 | 1 |
| Quinine | 1750 ± 85 | 1750 ± 90 | 1 |
| Cholate | 4450 ± 500 | 4450 ± 500 | 1 |
| Rhodamine 6G | 4.0 ± 0.5 | 4.0 ± 0.5 | 1 |
| Acridine orange | 950 ± 50 | 950 ± 50 | 1 |
| Ethidium bromide | 4.8 ± 0.3 | 4.8 ± 0.3 | 1 |

**a** Results shown are the average of three independent experiments with indicated standard error of the mean. $IC_{50}$ values were calculated from dose–response curves as shown in Fig. 3 and values correspond to the concentration of the toxic compound at which growth was inhibited by 50 %; **b** Ratio of $IC_{50}$ for the wild type over the *Δsmr1* mutant.

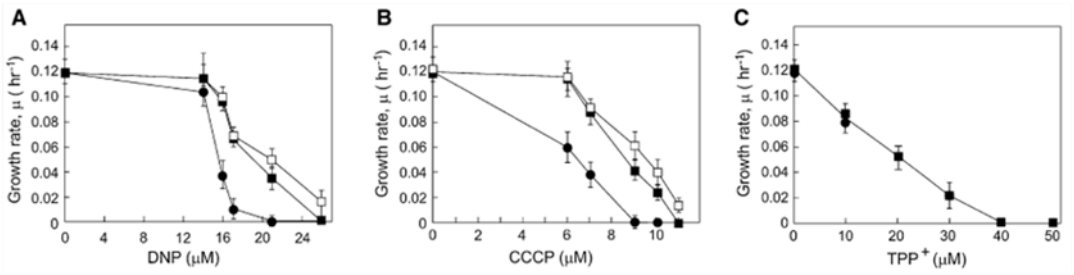


**Fig. 3** Sensitivity of the *S. acidocaldarius* MR31 and *Δsmr1* strains to diverse toxic compounds. *S. acidocaldarius Δsmr1* transformed with plasmid pSVA1450 (filled circle) or pSVA*smr1* (unfilled square) and the parental MR31 strain (filled square) were grown in Brock's medium in the presence of increasing concentrations of DNP (**a**) or CCCP (**b**). In addition, growth of the *S. acidocaldarius Δsmr1* (filled circle) and MR31 (filled square) strain was measured in the presence of tetraphenylphosphonium ion ($TPP^+$) (**c**). The growth rate is plotted as a function of the drug concentration.

many these micro-organisms thrive in hostile environments where there is a need for cellular protection against toxic molecules. Archaea are generally considered to be relatively robust due to their long evolutionary history and specific adaptations to extreme environmental conditions, and this possibly already renders these organisms less sensitive to toxic compounds (Dridi *et al.,* 2011). Nevertheless, the impact of MDR transporters on resistance in archaea has only marginally been studied. Bioinformatics analysis revealed the presence of both primary and secondary putative multidrug transporters in *S. acidocaldarius*. Various ABC transporters are annotated as MDR transporters, but none of such archaeal ABC transporters have been associated with drug resistance before (Lee *et al.,* 2007; Albers *et al.,* 2004). *saci_2123* specifies an ABC half-transporter consisting of a transmembrane domain and an ATP-binding domain fused as one polypeptide. Such proteins are typically involved in MDR or other diverse functions in bacteria and eukaryotes specifying a secretion activity (Holland *et al.,* 2003; Higgins 2007; Lubelski *et al.,* 2007). In addition, a homolog of Saci_2123 was found in related *Sulfolobales* and other Crenarchaeota that all harbor only one 'half-transporter' gene. These proteins share a unique feature as they all harbor a C-terminal DUF1854 domain. However, so far the function of this domain is unknown.

The functional analysis of Saci_2123 is consistent with a role in cellular protection against toxic compounds. First, the expression of *saci_2123* is induced upon drug exposure as shown for the uncoupler dinitrophenol. Second, a strain containing a deletion of the *saci_2123* gene is susceptible against a range of drugs. Third, plasmid-based expression of *saci_2123* reverses this drug sensitivity, and cells become even slightly more resistant than the wild type strain. These data suggest that also in the domain of archaea, MDR transporters fulfill a role in resistance and this will contribute to the ability of these organisms to withstand extreme environmental conditions. In this respect, previously, a protein of the small multidrug resistance (SMR) family of transporter proteins, Hsmr, was identified in *Halobacterium salinarum* (Ninio and Schuldiner, 2003). The gene was cloned and expressed in *E.coli* providing resistance to ethidium bromide and acriflavine. Indirect experimental evidence suggests the involvement of an ABC-MDR transporter in *H. volcanii* (Kaidoh *et al.,* 1996). An anthracycline-resistant mutant was found to transport rhodamine 123 more efficiently than the wild type strain, and this feature was reduced by ABC-proteins modulators and activated by cytotoxic compounds or metabolites, but the activity was not associated with a particular gene.

We also investigated the sensitivity of wild type and knockout strains to different toxic compounds, including two fluorescent dyes, ethidium bromide and Hoechst 33342. These compounds are readily permeate the cell membrane of cells and upon intercalating with DNA, their fluorescence increases (Lalande *et al.,* 1981; van den Berg *et al.,* 2005), which makes them ideal fluorescent probes to assessing the MDR transport activity. However, neither appears to be a specific substrate of Smr1, and also no transport activity of these compounds could be demonstrated in *S. acidocaldarius* (unpublished data).

In conclusion, the Smr1 (Saci_2123) protein represents the first identification and initial characterization of an ABC multidrug efflux pump from archaea for which a role in drug resistance could be demonstrated experimentally. This system found in Crenarchaeota is involved in resistance to diverse structurally and functionally dissimilar toxic substrates, and may contribute to cellular resistance in natural environments such as acidic high temperature volcanic springs.

## ACKNOWLEDGEMENTS

This work was supported by a China Scholar Council (CSC) grant to N.Y.

# 4

# BINDING PROTEIN-DEPENDENT IRON-UPTAKE IN THE CRENARCHAEON *SULFOLOBUS ACIDOCALDARIUS*

Nuan Yang and Arnold J. M. Driessen

## ABSTRACT

Iron is a vital metal for organisms but not always readily available from environment. Therefore, cells dependent on specific acquisition mechanisms that release the iron ion from the environment in a form that can readily taken up, such as the secretion of extracellular siderophores. Here we have examined the mechanism of iron uptake by the thermoacidophilic archaeon *Sulfolobus acidocaldarius* that grows at 80-85 °C and pH 2-3. At this low pH, iron ions are more soluble and likely to be more readily available. The genome of *S. acidocaldarius* specifies three putative iron substrate-binding proteins. Expression of these genes is regulated by iron, and their deletion affected growth under iron-limiting and rich conditions, which could be attributed to a defect in the uptake of iron. These data suggest that iron uptake in *S. acidocaldarius* is binding protein dependent.

# INTRODUCTION

Iron is typically the most abundant metal ion in cells (Vyllinniskii *et al.,* 2012) and required for multiple metabolic functions, such as, oxygen transport, tricarboxylic acid cycle activity, DNA synthesis (Drazek *et al.,* 2000), and electron transport (Conrad *et al.,* 2000; Kaplan 2009; Hentze *et al.,* 2010; Cyert and Philpott, 2013). Iron is usually not freely available in the environment but in complex with other molecules or as non-soluble salt and present in the oxidized form. This necessitates highly efficient mechanisms for iron acquisition from the environment (Weinberg 1997). Iron mainly exists in two redox states: the reduced $Fe^{2+}$ and the oxidized $Fe^{3+}$. The ferrous ion has a much better solubility, but only exists under anoxic or at very low pH conditions. At a neutral pH and in an oxidative environment, iron is predominantly present in the ferric ion ($Fe^{3+}$) stable form, which is extremely poorly soluble, rendering it biologically unavailable (Spiro and Saltman, 1969). Although iron is necessary for growth, a high internal iron concentration also makes cell vulnerable to oxidative damage. Therefore, it is of importance to tightly control the intracellular iron homeostasis (Andrews *et al.,* 2003).

Microorganisms are equipped with different strategies to solubilize, transport and store ferric or ferrous iron for maintaining an optimal intracellular iron concentration. Of the various systems of iron acquisition and uptake, there are three different groups of ATP binding cassette (ABC) transporters that are of particular interest, i.e., the siderophore/heme/vitamin B12 type, the ferric iron type and the metal ion type, respectively. These binding proteins are associated with ABC transport systems that allow the free or sequestered iron to enter the cell where it is subsequently released and if necessary, reduced to the ferrous ion form.

The structural features of ABC transporter system are conserved across the phylogenetic spectrum (Wandersman and Stojiljkovic, 2000; Genco and Dixon, 2001; Higgins 2001; Smith *et al.,* 2002). These systems operate in the uptake or secretion of a wide variety of substrates. However, uptake systems employ an additional and specific substrate binding proteins (Boos 1974; Higgins *et al.,* 1986; Dean *et al.,* 1989) that binds the ligand selectively and with high affinity. Although all ABC systems have a common origin, the three different iron uptake related ABC transport families are essentially distinguished on the basis of the structure of the binding protein. The structures of a multitude of binding proteins have been solved, with the maltose-binding protein (MBP) being the most widely studied in both bacteria and archaea (Higgins 1986, 1992; Elizabeth *et al.,* 2002). Binding proteins in general contain two independent globular domains linked by a flexible hinge with the ligand-binding domain at the interface of these two domains. Despite low overall sequence identities, substrate-binding proteins can be subdivided into two groups based upon the number of interdomain connections (Quiocho and Ledvina, 1996). More recently, a third class was identified wherein the two domains are bridged by a single R-helical segment. This class includes the vitamin $B_{12}$ binding protein BtuF (Borths *et al.,* 2002; Karpowich *et al.,* 2003) and the iron-hydroxamate binding protein FhuD (Borths *et al.,* 2002) of *E.coli*. These type-III binding proteins are thought to be more restricted in their domain movements and possess a more rigid interdomain linker than type I and II binding proteins.

Iron acquisition in bacteria has been extensively studied but little information is available on iron transport in archaea, in particular, the crenarchaea. Only in the halophilic *Halobacterium* ssp., siderophore production and iron uptake were studied in some detail (Dave *et al.,* 2005; Hubmacher *et al.,* 2007). In the present study, we have investigated the role of binding proteins in iron acquisition by *Sulfolobus acidocaldarius*. This thermoacidophile grows at low pH (pH 2.5) and

thus iron solubility will be less problematic compared to the less acidic environment of soils and other habitats. The results suggest the involvement of multiple metal ion binding proteins in iron acquisition.

## MATERIALS AND METHODS

**Strains and growth conditions**

*S. acidocaldarius* MR31 (Reilly and Grogan, 2001) as wild type strain and all marker-less deletion mutants were grown aerobically with or without $FeCl_3$ at 78˚C in Brock's medium (Brock *et al.*, 1972), supplemented with 0.2% (wt/vol) tryptone, 20 µg/ml uracil. The pH was adjusted to 3.5 with sulfuric acid, and when necessary 0.2% (wt/vol) N-Z-Amine, 0.2% (wt/vol) xylose, 50 mg/ml 5-fluoroorotic acid (5-FOA) and 0.64% Gelrite (GmbH) were added. Growth in the presence of xenosiderophore was measured at a low iron concentration complexed with the compounds indicated. Complexes were prepared as a stock solution of 15 µM $Fe^{3+}$ with 15 µM citric acid or 7.5 µM dihydroxy benzoic acid. All glassware for the iron-limited experiments was pre-treated with methanol/KOH, HCl and EDTA for 4 hours to ensure that residual iron comes from the metal ion present as a contaminant in the chemicals used to prepare the growth media.

**Construction of the deletion mutants**

To investigate the role of the three metal binding proteins, different deletion mutants were constructed. To inactivate the target genes *saci_1220*, *saci_2323* and *saci_1488*, the up- and down-stream flanking regions of the gene of interest were cloned (primer sets indicated in Table S1) and fused into the gene targeting vector pΔ2pyrEF (Wagner *et al.,* 2009). The resulting plasmids were transformed into *S. acidocaldarius* MR31 and individual deletion mutants were isolated as described previously (Ellen *et al.,* 2010). After removal of the *pyrEF* selection marker, the single mutant *Δsaci_1220* was used as a template to generate adouble (*Δsaci_1220_2323*) and triple (*Δsaci_1220_2323_1488*) mutant strain.

**Growth curves**

Pre-cultures of the wild-type and mutants strains were spread on Brock's plates, and incubated for 6 days at 78°C. Individual colonies were then grown in liquid culture without $FeCl_3$ until the early log phase, diluted 50-fold into fresh iron rich or limited medium. Growth of cells was continued and monitored by measuring the optical density at 600 nm.

**RNA isolation and transcriptional analyses**

Total RNA of the wild type and mutant strains at same time points in different iron conditions mediums was isolated with TRIzol (Invitrogen), with additional DNase treatment using the Turbo DNA-free kit (Ambion). cDNA was synthesized by iScriptcDNA synthesis kit(Bio-Rad). The final concentration of total RNA was measured with a NanoDrop ND-1000. Gene expression was quantified by real-time quantitative PCR (qPCR) as described previously (Ellen *et al.,* 2011), using the primers indicated in Table S2. All primers were designed such that fragments of 290bp were obtained. The gene expression levels were analyzed with a Mini Opticon system (Bio-Rad) using the Bio-Rad CFX manager software.

**Iron uptake assays**

*S. acidocaldarius* cells were cultured to late exponential phase in iron-limited Brock's medium, harvested by centrifugation (15 min, 3500 rpm,) and washed three times with iron-free Brock's medium. Pelleted cells were finally resuspended in the same medium to an optical density at 600 nm of around 3. Cell suspensions (1 ml) were collected for protein determination. A $^{59}FeCl_3$ stock solution (80 μM final concentration) was prepared by incubating 2 μl $^{59}FeCl_3$ (Perkin-Elmer, 1857.43 MBq/mg) to 198 μl HCl (10 mM) at room temperature for 30 min before adding it to the uptake assay at a final concentration of 40 nM. Transport assays were performed with 0.2 ml of the cell suspensionat 60 °C with shaking in a gyratory shaker. At various time points, uptake was stopped by adding 2 ml of ice-cold iron-free Brock's medium, filtered through nitrocellulose membranes (0.45 μm) that were washed with 5 ml of iron-free Brock's medium and with 5 ml of cold 0.1 mM LiCl. The membranes were dissolved in 2 ml of scintillation liquid (Perkin-Elmer), and cell associated radioactivity was measured using a liquid scintillation counting (LS6500, Beckman, Munich, Germany). The first sample (t = 0) was measured directly after the addition of the radiolabeled $^{59}Fe$ and used to quantify binding as control. To examine the kinetic parameters of iron transport, the iron ion concentration was varied between 0 and 100 μM, and the initial rates of uptake were determined after 5 minutes of uptake. For metal ion competition studies, metal ions were added to the $^{59}FeCl_3$ solution as chloride salt 5 min prior to uptake experiment. Data are the result of at least three replicate assays. To analyze the data, nonlinear regression analysis was performed using Origin 7 graphing software.

**Miscellaneous methods**

For protein determinations, cells were harvested by centrifugation (13,000 rpm, RT, table centrifuge), washed twice with iron-free Brock's medium, and resuspended in 50 mM Tris-HCl at pH 7.2. Cells were stored at –20 °C overnight and disrupted on ice with a Branson sonifier. The total amount of protein was measured with a Lowry based protein assay (DC Protein Assay, BioRad).

# RESULTS

**Bioinformatics analysis of iron uptake by *S. acidocaldarius***

Bioinformatic search of the S. *acidocaldarius* genome database using bacterial iron transporters and subsequent sequence analysis identified several genes with a potential function in iron acquisition. The *sac_1220*, *saci_1221* and *saci_1222* genes are localized in an operon and possibly encode a metal ion uptake system that belongs to the superfamily of ABC transporters. *saci_1222* encodes the ATP-binding component, and *saci_1221* encodes a membrane protein that may act as the permease domain. *saci_1220* is predicted to encode a helical backbone metal receptor belonging to the TroA-like superfamily. This family of proteins has been shown to function in the ABC transport of ferric siderophores and metal ions such as $Mn^{2+}$, $Fe^{3+}$, $Cu^{2+}$, and/or $Zn^{2+}$. Their ligand-binding sites are formed in the interface between two globular domains linked by a single helix and are structurally similar, to the β-subunit of the nitrogenase molybdenum-iron protein MoFe. These genes are annotated as a vitamin $B_{12}$ uptake system but no functional studies have been performed to validate this annotation. *saci_2323* encodes another member of this binding protein family but

does not localize to the gene cluster encoding the aforementioned ABC transporter. A further gene, *saci_1488*, encodes a protein that belongs to the LbetaH superfamily of binding proteins and that shows homology to ferripyochelin binding proteins that play a role in iron acquisition using the chelator pyochelin. Iron ions are incorporated into iron-sulfur (Fe-S) clusters, which constitute integral functional groups of many enzymes (Bandyopadhyay *et al.*, 2008). The *S. acidocaldarius* genome also contains genes specifying the biogenesis of Fe-S clusters, the so-called SUF system that includes an ATPase SufC (Saci_1388) and permease domain (Saci_1389, Saci_1390) likely involved in the delivery of iron for the assembly of FeS clusters, which is a specific function and not an activity generally involved in iron acquisition. Two further genes (*saci_1820*, *saci_2094*) encode possible $Mn^{2+}$ and $Fe^{2+}$ transporters belong to the NRAMP family (Inorganic ion transport and metabolism). It should be stressed that the above genes are found in all *Sulfolobus* species sequenced to date implying that there might be multiple mechanisms present for iron acquisition.

**Table 1** Bioinformatic analysis of potential transporters of *S. acidocaldarius* with a putative role in iron uptake and delivery.

| GenBank accession No. | Predicted function | Gene product |
|---|---|---|
| Saci_1220 | Ferric iron acquisition | Metal ion binding protein |
| Saci_1221 | | ABC permease membrane protein |
| Saci_1222 | | ABC ATPase domain |
| Saci_2323 | | Metal ion binding protein |
| Saci_1488 | | Ferripyochelin binding protein |
| Saci_1820 | Free ferrous iron acquisition | Transporter of NRAMP family |
| Saci_2094 | | Transporter of NRAMP family |
| Saci_1388 | Iron-sulfur cluster formation | Fe-S assemblyATPase SufC |
| Saci_1389 | | Fe-S assembly permease membrane protein |
| Saci_1390 | | Fe-S assembly permease membrane protein |

**Growth of *S. acidocaldarius* under iron-depleted conditions**

Brock's medium normally contains 75 μM $FeCl_3$ in addition to the $Fe^{3+}$ that originates from contaminants and that is present in glassware. When no $Fe^{3+}$ is added, still growth of *S. acidocaldarius* is observed but at a 2-fold reduced rate (Fig. 1). In addition, we examined if growth of S. *acidocaldarius* under iron-limiting conditions is promoted by iron chelators belonging to two classes of siderophores: the catecholate siderophore dihydroxy benzoic acid, and the carboxylate-type siderophore citrate. Neither of these siderophores significantly promoted growth both in the presence or absence of 15 μM $FeCl_3$ (Fig. 2).

**Iron transport by *S. acidocaldarius***

To investigate the characteristics of iron uptake in *S. acidocaldarius*, uptake experiments with $^{59}Fe^{3+}$ were conducted. For this purpose, the MR31 strain was grown in Brock's medium under

iron-limiting conditions up to the late exponential state. Interestingly, the uptake of $^{59}Fe$ was almost 2-fold higher (Fig. 3) when cells were grown in Brock's medium, indicating that iron-depletion does not stimulate the uptake of $^{59}Fe^{3+}$.

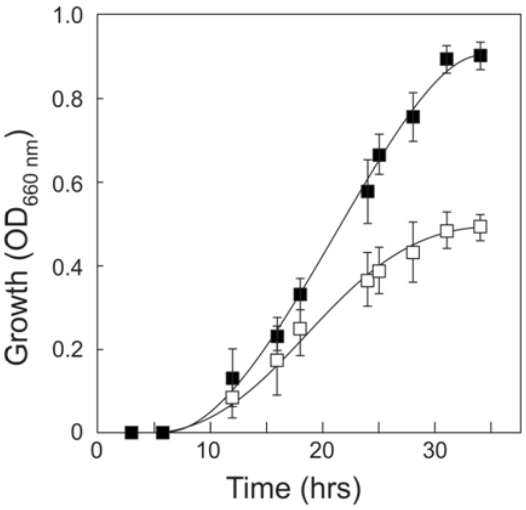


**Fig. 1** Growth of the wild-type strain MR31 under iron-limiting and iron-sufficient growth conditions. Symbols represent: filled squares, with $Fe^{3+}$ supplement; open squares, without $Fe^{3+}$ supplement. Each value in the curves is the mean of three independent replicates. The growth of all strains is comparable.

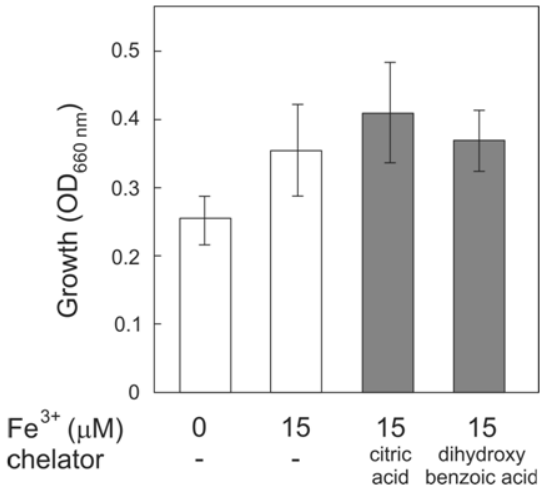


**Fig. 2** Effect of various xenosiderophores on the growth of *S. acidocaldarius* on Brock's medium with the indicated concentration of iron.

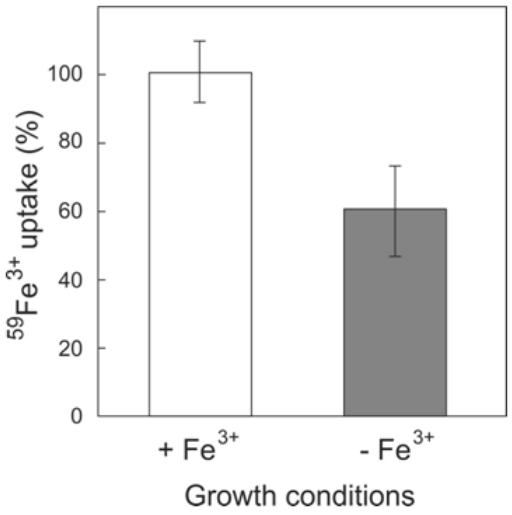


**Fig. 3** Uptake of $^{59}Fe^{3+}$ by *S. acidocaldarius* grown in Brock's medium in the presence or absence of 75 μM $FeCl_3$. Uptake was recorded after 5 minutes, and expressed in % of the control (iron-rich conditions). Data shown is the average of 3 independent experiments with the indicated standard error of the mean.

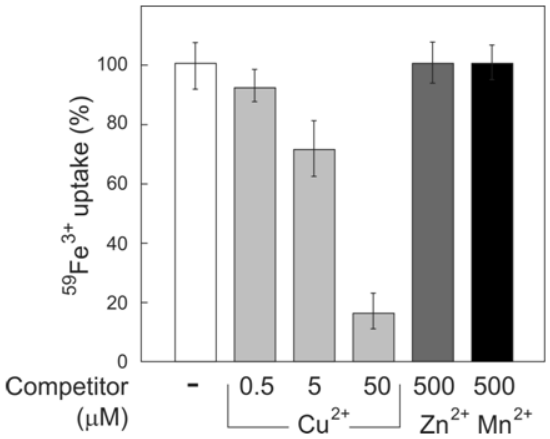


**Fig. 4** Competition of $^{59}Fe^{3+}$ uptake by other metal ions. Wild-type cells were incubated with different concentrations of indicated metal ions, respectively. Uptake was measured for 5 minutes and expressed as percentage of $^{59}Fe^{3+}$ uptake relative to values without competitor (100%). Data shown is from three independent experiments with the indicated standard of the mean.

The uptake of $^{59}Fe^{3+}$ showed a typical Michalis-Menten type of kinetics with a $V_{max}$ and $K_m$ value of 26.34 pmol $min^{-1}$ $mg^{-1}$, and 4.68 μM $Fe^{3+}$, respectively. To investigate the specificity of $^{59}Fe^{3+}$ uptake, competition experiments were performed using a set of other metal ions. $Cu^{2+}$ efficiently competed for $Fe^{3+}$ uptake, whereas high concentrations of $Zn^{2+}$ and $Mn^{2+}$ were ineffective as competitors (Fig. 4).

**Expression of putative metal ion binding protein encoding genes under iron-limiting growth conditions**

To investigate the role of the three identified binding proteins (Saci_1220, Saci_1488, and Saci_2323) in iron uptake, their expression profiles were determined with *S. acidocaldarius* M31 cells grown in complete Brock's medium and under iron-limited conditions. Herein, total RNA was collected from cells grown at same stages of growth (mid exponential), and expression was analyzed by quantitative real-time PCR (qPCR). The transcription of *saci_1220* and *saci_1488* was significantly enhanced when cells were grown under iron-limiting conditions (Fig. 5), whereas *saci_2323* seems invariant to low iron.

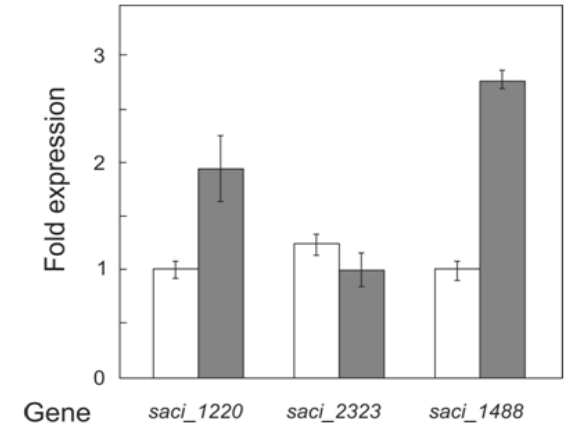


**Fig. 5** Expression levels of the indicated putative metal binding protein genes from *S. acidocaldarius* grown under iron-sufficient (white bars) and -limiting (gray bars) conditions. Expression was determined by real-time quantitative PCR using the *secY* gene as a reference.

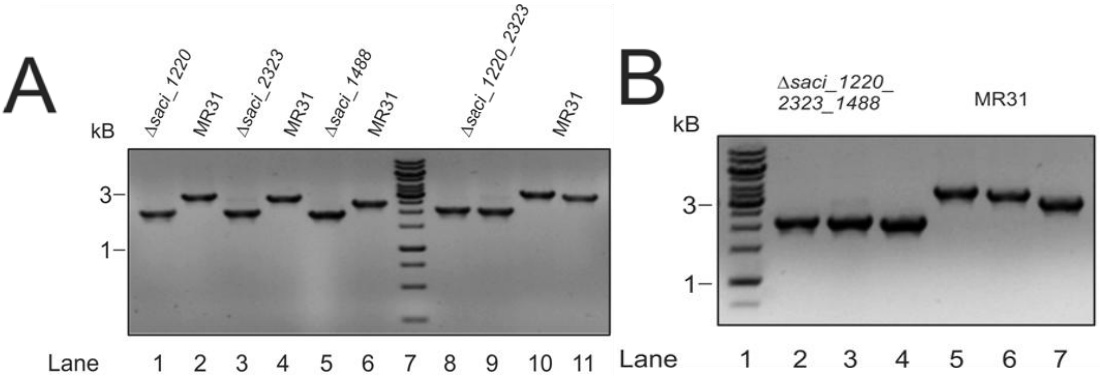


**Fig. 6** Verification of the gene inactivation of three putative metal ion binding proteins. PCR analysis of individual strains for the absence of the *saci_1220* gene (lane 1a), *saci_2323* gene (lane 3a), *saci_1488* gene (lane 5a), double knockout *saci_1220_2323* [*saci_1220* gene (lane 8a), *saci_2323* gene (lane 9a)], triple knockout *saci_1220_2323_1488* [*saci_1220* gene (lane 2b), *saci_2323* gene (lane 3b), *saci_1488* gene (lane 4b)] , and presence of the *saci_1220* gene (lane 2a), *saci_2323* gene (lane 4a), *saci_1488* gene (lane 6a), double knockout *saci_1220_2323* [*saci_1220* gene (lane 10a), *saci_2323* gene (lane 11a)], triple knockout *saci_1220_2323_1488* [*saci_1220* gene (lane 5b), *saci_2323* gene (lane 6b), *saci_1488* gene (lane 7b)], in the genomic DNA derived from the wild-type and single, double and triple mutant strains of *S. acidocaldarius*, respectively. The PCR products were subjected to electrophoresis in 1% agarose.

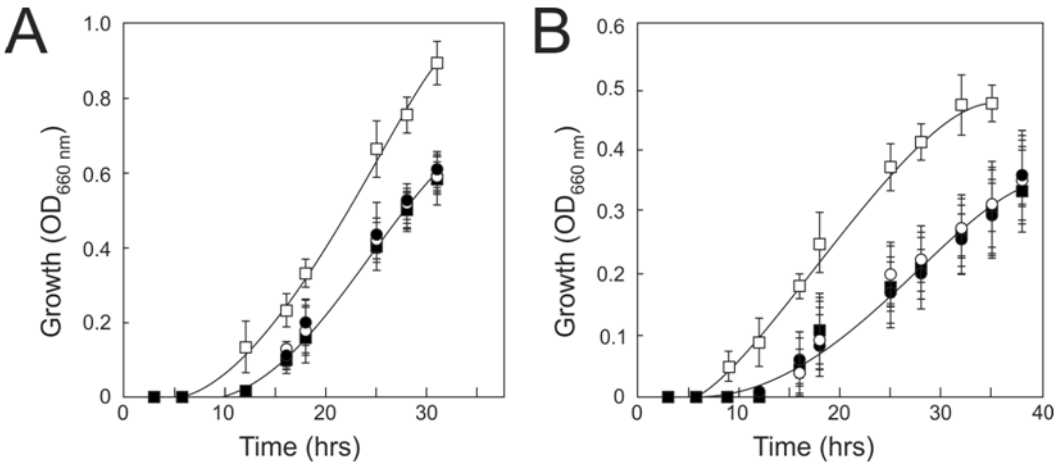


**Fig. 7** Growth of *S. acidocaldarius* strain MR31 and indicated mutants under iron-sufficient (**A**) and -limited (**B**) growth conditions. Symbols represent: open squares, wild-type; open triangle, *Δsaci_1220*; open rhombus, *Δsaci_2323*; open circles, *Δsaci_1488*; filled triangle, *Δsaci_1220_2323*; filled circles, *Δsaci_1220_2323_1488*. Each value in the curves is the mean of three independent replicates.

**Deletion of the putative metal-ion binding protein genes and effect on growth and iron uptake**

To further examine the role of the three putative metal ion binding proteins, their genes were deleted from the chromosome of *S. acidocaldarius* strain MR31. Herein, individual gene deletions were generated, i.e., *Δsaci_1220, Δsaci_2323* and *Δsaci_1488,* as well as a double (*Δsaci_1220_2323*) and triple (*Δsaci_1220_2323_1488*) deletion mutant. Chromosomal deletions were verified by PCR analysis (Fig. 6). The strains were grown on normal Brock's medium and iron-limited medium. With all mutants, growth on normal Brock's medium was reduced compared to that of the parental strain. Remarkably, the growth phenotypes of the single, double and triple mutants were very similar (Fig. 7A). Under iron-limited conditions, the growth of the single mutants was dramatically reduced and again similar to that of the double and triple mutant (Fig. 7B). These data suggest a role of these three binding proteins in iron acquisition, but also indicate that other mechanisms must prevail, as growth of the triple mutant under iron-limited conditions was not completely abolished.

Next the mutants were tested for the uptake of iron. For this purpose, cells were grown on iron-free Brock's medium. Uptake was assessed with 40 nM $^{59}Fe^{3+}$. As compared to the wild-type strain, uptake of iron was reduced by about 30% in the *Δsaci_1220* and *Δsaci_2323* strains, whereas a similar result was obtained with the *Δsaci_1220_2323* double deletion. A stronger deficiency was observed with the *Δsaci_1488* strain and the triple deletion showing a more than 50% reduction in iron uptake (Fig. 8).These observations indicate each of the iron binding proteins contribute to the uptake of iron but further demonstrate that additional mechanisms exist for the uptake of iron.

**Expression of putative metal ion binding proteins in the deletion strains**

Since the growth studies and uptake experiments suggests that all three binding proteins contribute to iron uptake, we also examined the expression levels of the binding protein genes in the various deletion strains. One may argue that upon deletion of one gene, elevated expression of

another gene (specifying either another binding protein or another iron transporter) could compensate for the loss of activity. The mutant strains were grown on iron-limited medium and the expression levels were compared relative to the *S. acidocaldarius* parental strain that was grown under identical conditions. The level of *saci_1220* expression increased more than 2-fold in the *Δsaci_2323* and *Δsaci_1488* cells. Also, the *saci_1488* expression increased in the *Δsaci_2323* cells, while the expression levels of *saci_2323* did not change in the various deletion mutants (Fig. 9). These data indeed suggest that compensatory expression mechanisms are present to accommodate the deletion strains for the deficiency in $Fe^{3+}$ uptake. This partially explains the lack of the strong growth phenotypes in the individual deletion strains.

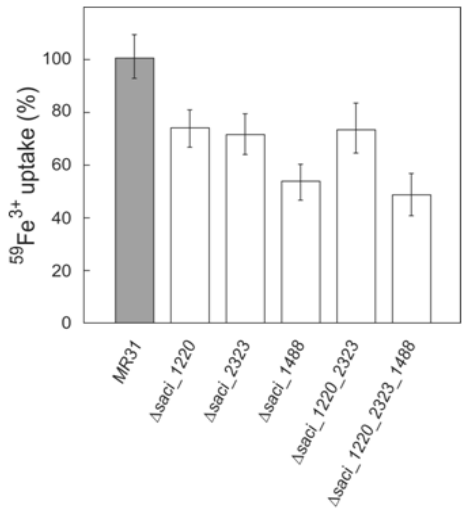


**Fig. 8** Uptake of $^{59}Fe^{3+}$ by *S. acidocaldarius* and the indicated deletion strains. Uptake was measured for 5 minutes and expressed as percentage of $^{59}Fe^{3+}$ uptake relative to the wild type (100%). Data shown is from three independent experiments with the indicated standard of the mean.

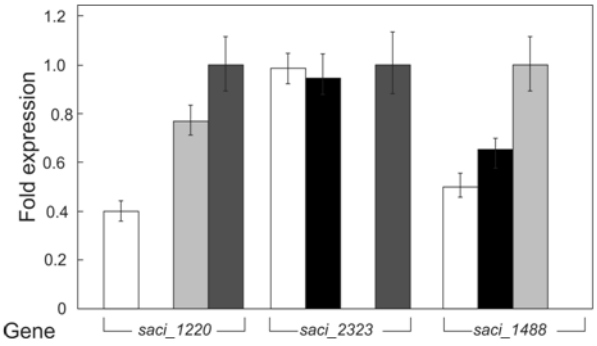


**Fig. 9** Expression levels of the indicated putative metal binding protein genes in the indicated *S. acidocaldarius* strains grown under iron-limited conditions. Bars designate the expression of the indicated gene in: wild-type strain (white); *Δsaci_1220* (black); *Δsaci_2323* (light gray) and *Δsaci_1488* (dark gray). Expression was determined by real-time quantitative PCR using the *secY* gene as a reference.

## DISCUSSION

Here we have presented an initial study on the mechanism of iron transport in *S. acidocaldarius*. Our data suggest that binding protein dependent transport mechanisms are operational in this organism but also demonstrates that additional mechanism must be operational as the removal of all three binding protein genes does not completely block iron uptake. Consistent with other acidophiles microorganisms, only a small number of putative iron transporter genes are found and no genes for siderophore synthesis are reported in *S. acidocaldarius* genome. Also siderophores could not be detected in culture supernatants (data not shown) while our data shows that the addition of two xenosiderophores from different siderophore-classes does not promote growth or uptake.

In most iron transport systems studied so far, uptake requires a periplasmic-binding protein as well as an ABC transporter. Three putative iron uptake transporter binding proteins were identified in this study where only one seems to cluster in the chromosome with an ABC iron uptake system. Two of the putative iron binding proteins are up-regulated when cells were grown

in iron-limited medium, which indirectly links them to iron uptake. Therefore, different mutants were constructed with a single, double and triple deletion of the binding protein genes. However, deletion of either binding protein gene caused a growth defect both in iron-defect and rich medium as compare with their parent strain. Remarkably, the double and triple deletion did not strengthen the phenotype indicating that other mechanisms of iron uptake must also be present in *S. acidocaldarius*. Similar results were obtained with direct $^{59}Fe$ uptake experiments supporting the notion of additional iron uptake mechanisms. Also in the single deletion mutants, compensatory gene expression levels are observed possibly counteracting the defect introduced by the single deletions. The triple mutant only shows a slightly strong growth and uptake defect, and we hypothesize it is due to compensatory mechanisms on the expression of other transporters possibly those causing the background uptake levels. Possibly, this activity involves the transporter genes identified of the NRAMP family but this was not further addressed experimentally. So far our data does not support the presence of siderophore-based mechanisms. Like in other acidophilic microorganism such as *Leptospirillum*, this may not be required because of the acidic growth environment that supports a high ferric iron solubility. Future studies should assess the role of additional transport mechanisms in the uptake of iron to map the cellular mechanism of iron acquisition.

## ACKNOWLEDGEMENTS

Thanks to M. Blaauw and Erik J. Bunskoeke for help with the liquid scintillation counting, Lito Monti for help with the data analysis.

# 5

# REGULATION OF ARCHAELLA EXPRESSION BY THE FHA AND VON WILLEBRAND DOMAIN-CONTAINING PROTEINS ARNA AND ARNB IN *SULFOLOBUS ACIDOCALDARIUS*

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*Molecular Microbiology* (2012) 86:24–36

## ABSTRACT

The ability of microorganisms to sense and respond to sudden changes in their environment is often based on regulatory systems comprising reversible protein phosphorylation. The archaellum (former: archaeal flagellum) is used for motility in Archaea and therefore functionally analogous to the bacterial flagellum. In contrast with archaellum-mediated movement in certain members of the Euryarchaeota, this process, including its regulation, remains poorly studied in crenarchaeal organisms like *Sulfolobus* species. Recently, it was shown in *Sulfolobus acidocaldarius* that tryptone limiting conditions led to the induction of archaella expression and assembly. Here we have identified two proteins, the FHA domain-containing protein ArnA and the vWA domain-containing protein ArnB that are involved in regulating archaella expression in *S. acidocaldarius*. Both proteins are phosphorylated by protein kinases in vitro and interact strongly in vivo. Phenotypic analyses revealed that these two proteins are repressors of archaella expression. These results represent the first step in understanding the networks that underlie regulation of cellular motility in Crenarchaeota and emphasize the importance of protein phosphorylation in the regulation of cellular processes in the Archaea.

# INTRODUCTION

All microorganisms have to withstand and respond to sudden changes in their environment. The availability of nutrient sources is sensed to regulate metabolic pathways and to detect nutrient limiting conditions. Therefore, perception of extracellular stimuli and their transduction in the cell is essential for survival of microorganisms. Chemotaxis sensory systems link the signal input to motility, allowing microorganisms to escape hostile environments. Chemotaxis systems have been well studied in Bacteria, in which signals sensed by a chemoreceptor are always transduced to a two-component signal transduction pathway, which modulates flagella movement upon binding of phosphorylated CheY-P to the flagellar motor switch protein FliM (Welch *et al.,* 1993; Dyer *et al.,* 2009). Among Archaea, only the Euryarchaeota possess such a bacterial-like chemotaxis system, which is best studied in *Halobacterium salinarum* (Rudolph *et al.,* 1995; Rudolph and Oesterhelt, 1996). However, the archaellum [former the archaeal flagellum (Jarrell and Albers, 2012)] is only functionally, but not structurally related to the bacterial flagellum, as it is evolutionary related to bacterial type IV pili. Two core subunits of the archaellum, the ATPase FlaI and the integral membrane protein FlaJ, are homologues of PilB and PilC in bacterial type IV pili (Pohlschroder *et al.,* 2011). Moreover, the processing of the archaellins, the structural subunits of the archaellum, prior to assembly is conserved in bacterial type IV pilins (Pohlschroder *et al.,* 2011). Therefore the final step in regulating archaella-mediated movement upon CheY-P interaction is envisioned to differ from the bacterial flagella system. In *H. salinarum* three proteins (two of the DUF439 family, CheM, and one of the HEAT_PBS family) were identified that bind the chemotaxis proteins CheY, CheD and CheC2 as well as the archaella proteins FlaCE and FlaD. Hence, these proteins seemingly connect the sensory system to the motility system in this organism (Schlesner *et al.,* 2009; Alatyrev *et al.,* 2010). In *Methanococcus jannaschii* and *M. maripaludis* archaella expression increased at low hydrogen concentrations and additionally was reduced during leucine limitation in *M. maripaludis* (Mukhopadhyay *et al.,* 2000; Hendrickson *et al.,* 2008). However, how archaella regulation is achieved in Crenarchaeota is not understood, as information about the regulatory pathways and the inducing conditions is not available. Homologues of chemotaxis proteins have not been identified in the Crenarchaeota so far. Recently, it was shown that in *Sulfolobus acidocaldarius*, one of the main crenarchaeal model organisms, tryptone limiting conditions led to the induction of archaella expression and assembly (Lassak *et al.,* 2012). However, the factors involved in regulation of this process are still unknown.

Forkhead-associated (FHA) domains were first described as modules occurring in forkhead transcription factors (Hofmann and Bucher, 1995). Since then, FHA domain-containing proteins were structurally and functionally analyzed in detail revealing a phosphopeptide-binding activity with a clear specificity towards p-Thr over p-Ser or p-Tyr (Durocher *et al.,* 2000; Pennell *et al.,* 2010). Because of these specific interactions FHA domains, which are found in all three domains of life, are important modules in phosphopeptide-mediated processes in the cell like signal transduction and DNA damage response pathways. Most studies on FHA domains were conducted in Eukaryotes or Bacteria, whereas 33 of these phosphopeptide-specific binding proteins are predicted in archaeal genomes (http://smart.embl-heidelberg.de/). FHA domain-containing proteins not only bind phosphopeptides, but can also be phosphorylated *in vitro* by eukaryotic-like protein kinases (ePKs) that are distantly encoded on the genome in *Mycobacterium tuberculosis* (Grundner *et al.,* 2005). A similar result was shown for the crenarchaeal *Sulfolobus tokodaii* FHA protein, which was phosphorylated *in vitro* by a Serine/Threonine PK and binds the archaellar

*flaX* promoter in a phosphorylation dependent manner (Wang *et al.,* 2010; Duan and He, 2011). Strikingly, in *Sulfolobus* spp. these FHA proteins always seem to be present in an operon with a gene encoding for a von Willebrand domain-containing protein (vWA). Primarily, the von Willebrand type A domain of about 200 amino acids was found in the von Willebrand factor, a protein secreted to the blood plasma in mammals (Sadler *et al.,* 1985). The most ancient vWA proteins are now known to be intracellular proteins. These proteins are encoded in all three domains of life and are involved in different processes like signal transduction, cell adhesion and migration (Colombatti *et al.,* 1993; Whittaker and Hynes, 2002). A common feature of vWA proteins is to form multi-protein complexes (Tuckwell 1999; Whittaker and Hynes, 2002). However, in archaea these domains have not been functionally characterized.

In this study we have analysed the role of the FHA protein ArnA and the vWA domain-containing protein ArnB in the regulation of the archaellum operon in *S. acidocaldarius*. Both proteins were phosphorylated by specific ePKs and interacted strongly *in vivo*. Genetic analyses showed that these two proteins are repressors of archaella expression. These results represent the first step in understanding regulatory networks in Crenarchaea and underline the importance of protein phosphorylation in cellular processes in the Archaea.

## RESULTS

**The *arn* cluster and its evolution**

Interestingly, each member of the *Sulfolobales* contains only one FHA protein in its genome, which displays an N-terminal predicted Zinc finger domain. In *S. acidocaldarius* the FHA protein is encoded in an operon with one of the three vWA-containing proteins that are present in the *S. acidocaldarius* genome (Fig. 1A). Later on we demonstrate that these two proteins are part of the *archaellum regulatory network* and term these proteins as ArnA and ArnB (Fig. 1A). A comparative genome analysis revealed that the *S. acidocaldarius arn* cluster is conserved across a number of *Sulfolobus* species (Fig. 1B). The gene cluster invariably encodes an FHA domain-comprising gene and one copy of a gene encoding a vWA domain-containing protein. In the *Sulfolobales* this cluster is flanked upstream by a second vWA protein encoded in the opposite direction. Additionally, a gene encoding a protein with a predicted protein phosphatase activity seems to be part of the gene cluster in some cases, but this is not observed for *S. acidocaldarius* and *S. tokodaii*, where these genes are encoded at a distinct region on the chromosome (*saci_0884* respectively *ST2367*) (Fig. 1B). The presence of two gene copies of the vWA domain-containing proteins is best explained by an ancient gene duplication event at the base of the *Sulfolobales*, followed by lineage-specific gene loss and/or horizontal gene transfer events in the branches leading towards *Metallosphaera*. The presence of the *arn* gene cluster in *Picrophilus torridus* is most likely the result of a horizontal gene transfer event given that this is the only species of the Euryarchaeota that contains the gene cluster (Fig. 1C).

Interestingly, a variant of the *arn* gene cluster seems to be present in certain members of the *Desulfurococcales* (*Pyrolobus fumarii* and *Staphylothermus* spp.). In which these gene clusters do contain the FHA- and vWA containing genes, the protein phosphatase-encoding gene seems to be displaced by an unrelated Serine/Threonine protein kinase of the Pkinase superfamily (PFAM00069) (Fig. 1B). The presence of related modules in more distant archaeal species suggests that FHA and vWA proteins represent ancient regulatory networks that operate in conjunction

with protein phosphatases and protein kinases.

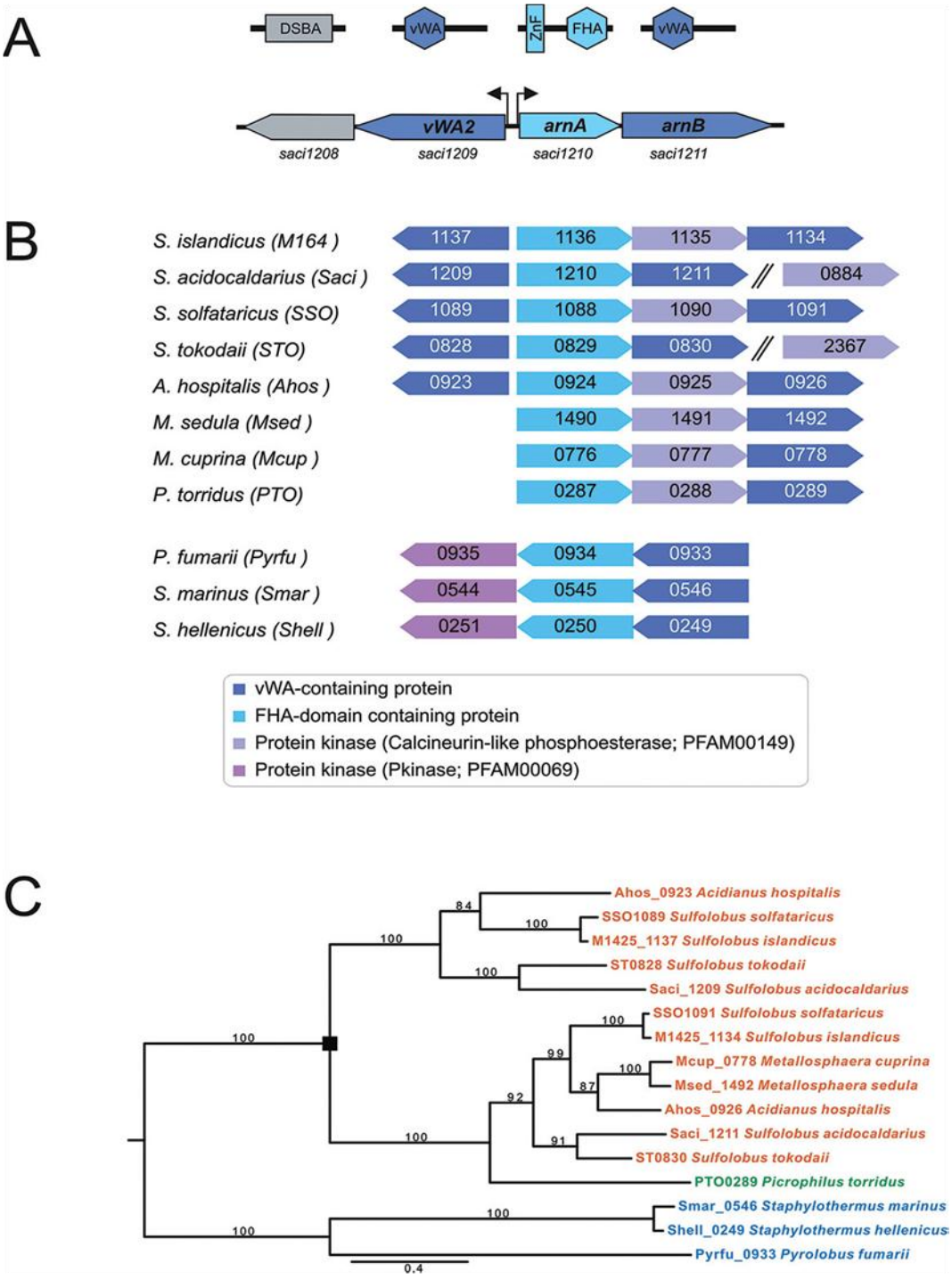



**Fig. 1** Comparative and phylogenetic analysis of the *arn* gene cluster and archaeal vWA proteins. **A**. Schematic overview of the *arn* gene cluster in *S. acidocaldarius*. Genes are indicated by arrows and the encoded protein architectures are depicted on top. The FHA domain-containing protein ArnA also incorporates an N-terminal zinc-finger domain. **B**. The *arn* gene cluster is conserved across a range of archaeal species and invariably comprises an FHA domain-containing gene and a vWA domain-containing gene in an operon structure, which is flanked upstream by a second copy of a gene encoding a vWA domain-containing protein in the *Sulfolobales*. In some species, the gene cluster additionally comprises a gene encoding a serine/threonine protein phosphatase. **C**. Phylogenetic tree

indicating that the distribution of the vWA domain proteins is best explained by an ancient gene duplication event at the base of the *Sulfolobales* (depicted with a filled square), followed by lineage-specific gene loss and/or horizontal gene transfer events in the branches leading towards *Metallosphaera* and the euryarchaeon *Picrophilus* (green font). Support values (based on 100 bootstrap replicates) are indicated for each branch, and the tree was rooted with vWA orthologues from the *Desulfurococcales* (blue font).

**Phosphorylation of ArnA and ArnB**

Both genes of the *arn* cluster of *S. acidocaldarius* and the upstream located vWA-encoding gene (*vWA2*, *arnA* and *arnB*) were successfully overexpressed in *Escherichia coli* and the proteins were purified (Fig. S1). The genomic organization to adjacent protein kinases and phosphatases in some species (Fig. 1B) suggested an involvement of these proteins in protein phosphorylation processes. Furthermore, FHA domain-containing proteins were shown to be phosphorylated by ePKs *in vitro* in *M. tuberculosis* and *S. tokodaii* (Grundner *et al.,* 2005; Wang *et al.,* 2010). Therefore *in vitro* phosphorylation studies were performed using [$\gamma$-$^{32}$P]-ATP (Figs 2 and 3). Two kinases of *S. acidocaldarius* (Saci_1193 and Saci_1694) were tested on autophosphorylation and phosphorylation of ArnA, ArnB and vWA2 since both kinases showed co-occurrence with ArnA in the STRING database (http://string-db.org/). Both kinases showed autophosphorylation activity, which is a common feature of ePKs (Fig. 2A and B, top). This autophosphorylation could be abolished in ATP binding-deficient mutants in which the conserved lysine residue in subdomain II was mutated to an alanine (Fig. 2A and B, top). Furthermore, the experiments revealed phosphorylation of all three proteins encoded in the *arn* cluster by the ePK Saci_1193, although ArnB clearly showed the strongest phosphorylation signal (Fig. 2A, top). Upon phosphorylation ArnB running behaviour changes on SDS-PAGE which was clarified with immunoblotting using specific $\alpha$-ArnB antibodies (Fig. 2A, middle). The second tested ePK, Saci_1694, specifically phosphorylated ArnB (Fig. 2B, top). Two mutants of ArnA (R132A and S146A) were also investigated for phosphorylation. These residues are highly conserved among the FHA domains and are involved in phosphopeptide binding (Fig. S2). Both mutants could still be phosphorylated, albeit the ArnA$_{S146A}$ showed a reduced phosphorylation signal (Fig. 3A). Moreover, phosphorylated ArnA and ArnB could be dephosphorylated by the *S. acidocaldarius* Ser/Thr phosphatase PPP (Fig. 3B and C).

**Interaction of ArnA and ArnB**

*arnA* and *arnB* are genomically located in a cluster and are both constitutively expressed in the exponential growth phase as well as in the stationary phase (data not shown). Since FHA domain-containing proteins as well as vWA containing proteins are well known to be involved in protein–protein interactions, the question arose if both proteins interact *in vivo*. Therefore, homologously expressed Strep/His-tagged ArnA and ArnB were purified by Ni-affinity chromatography. In the elution fraction of the purified ArnA$^{Strep/His}$, native ArnB was co-purified as confirmed by Western blot analysis (Fig. 4A) and mass spectrometry. In turn, when ArnB$^{Strep/His}$ was purified, native ArnA was co-eluted (Fig. 4A). This result demonstrates an *in vivo* interaction between the FHA domain-containing protein ArnA and the vWA domain-containing protein ArnB in *S. acidocaldarius*. Interestingly, in the elution fraction no vWA2 protein was detected by mass spectrometry, which confirms specificity of the binding between ArnA and ArnB. Heterologous

coexpression on a dual expression vector system (with ArnA Strep-tagged and ArnB His-tagged) in *E. coli* supports the previous result: a strong interaction between $ArnA^{Strep}$ and $ArnB^{His}$ (Fig. 4B).

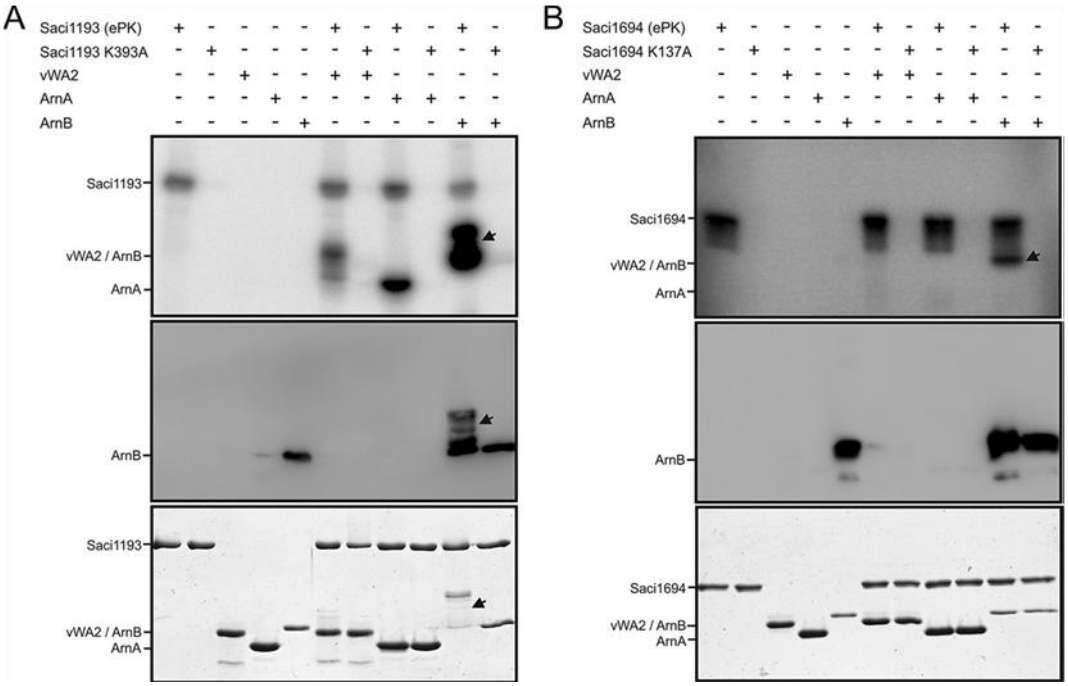



**Fig. 2** *In vitro* phosphorylation of ArnA, ArnB and vWA2 by the kinases Saci_1193 and Saci_1694. Proteins were incubated at 55°C for 30 min with [γ-$^{32}$P]-ATP. The reaction was stopped with 5X protein loading dye. **A**. The ePK Saci_1193 phosphorylated all three proteins (ArnA, ArnB and vWA2) *in vitro* (top). To show the upshift (arrow) of ArnB upon phosphorylation more clearly, immunoblotting was performed with specific α-ArnB antibodies (middle). This upshift is also visible on SDS-PAGE (bottom). **B**. The ePK Saci_1694 specifically phosphorylates ArnB (arrow). To confirm that the phosphorylation is on the height of ArnB and not an effect of the autophosphorylation smear of Saci_1694, immunoblotting was performed with specific α-ArnB antibodies (middle) after running SDS-PAGE (bottom).

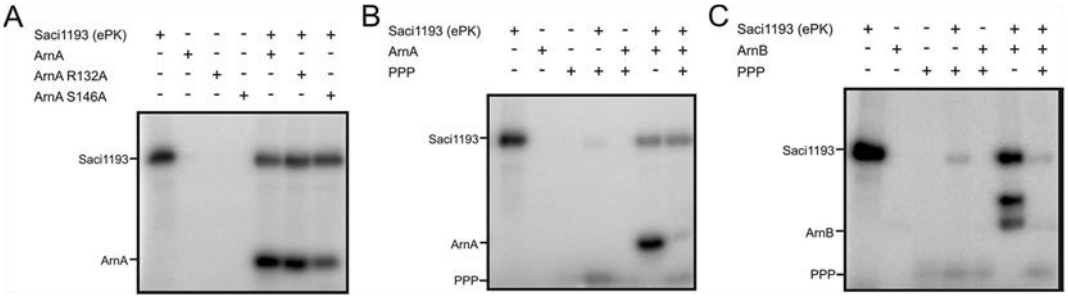



**Fig. 3** *In vitro* phosphorylation of ArnA mutants by the kinase Saci_1193 and dephosphorylation of ArnA and ArnB with the Ser/Thr phosphatase PPP. Proteins were incubated at 55°C for 30 min with [γ-$^{32}$P]-ATP. The reaction was stopped with 5X protein loading dye. **A**. Different mutants of ArnA, responsible for phosphopeptide interaction of the FHA domain (R132A and S146A), were tested for their phosphorylation behavior. **B**. ArnA could be dephosphorylated by addition of the Ser/Thr phosphatase PPP and incubation for another 10 min. **C**. Also ArnB showed a reduced phosphorylation signal upon addition of the Ser/Thr phosphatase PPP.

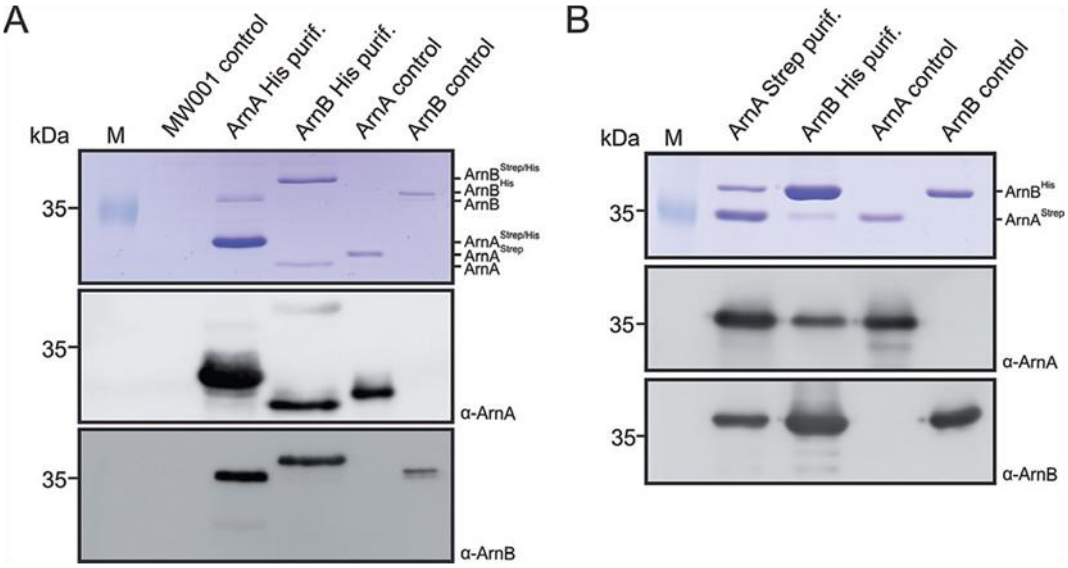



**Fig. 4** *In vivo* interaction of ArnA and ArnB. **A**. Coomassie-stained SDS-PAGE of the co-purification of ArnA and ArnB homologously expressed in *S. acidocaldarius*. Either ArnA or ArnB was expressed with a Strep/His tag and purified with Ni-affinity chromatography. The different running patterns of the proteins result from the different tags in comparison with the un-tagged native versions. In the elution fractions ArnA and ArnB were identified by specific antibodies using immunoblotting. The control proteins $ArnA^{Strep}$ and $ArnB^{His}$ were expressed in *E. coli*. **B**. Coomassie-stained SDS-PAGE of the co-purification of $ArnA^{Strep}$ and $ArnB^{His}$ from heterologous coexpression in *E. coli* by using either a Strep-Tactin column or a Ni-NTA column. In these fractions ArnA and ArnB were identified by specific antibodies using immunoblotting. M, marker.

**ArnA and ArnB repress archaellum gene expression**

To analyze the *in vivo* function of ArnA and ArnB, in-frame deletion mutants of *vWA2*, *arnA* and *arnB* were constructed in *S. acidocaldarius* MW001 using a single-cross-over pop in/pop out mechanism based on uracil auxotrophy. All deletion mutants were identified by PCR in comparison with the MW001 background strain and confirmed by sequencing (Fig. 5A). All three deletion mutants showed no defect in growth compared with the background strain MW001 (Fig. 5B). It was recently suggested that the *S. tokodaii* ArnA binds in its unphosphorylated state to the *flaX* promoter of the archaellar gene cluster (Duan and He, 2011), indicating a regulatory role in archaellum assembly (Fig. 6A). In *S. acidocaldarius* it was shown that tryptone starvation induced expression of the archaellum and therefore of the structural subunit FlaB as well as of the archaellar component FlaX (Lassak *et al.*, 2012). To test a regulatory role of ArnA and ArnB in archaellum expression, MW001, the *ΔvWA2*, *ΔarnA* and *ΔarnB* deletion strains were subjected to tryptone starvation and the levels of FlaB and FlaX were detected by Western blot analysis. While the expression levels of both FlaB and FlaX, were the same in the wild type as in the *ΔvWA2* deletion strain, their accumulation was significantly higher induced in *ΔarnA* and *ΔarnB* deletion strains (Fig. 6B). The double-deletion strain *ΔarnAΔarnB* showed the same phenotype as the single-deletion mutants (data not shown). These results imply that ArnA and ArnB are repressors of archaellum expression.

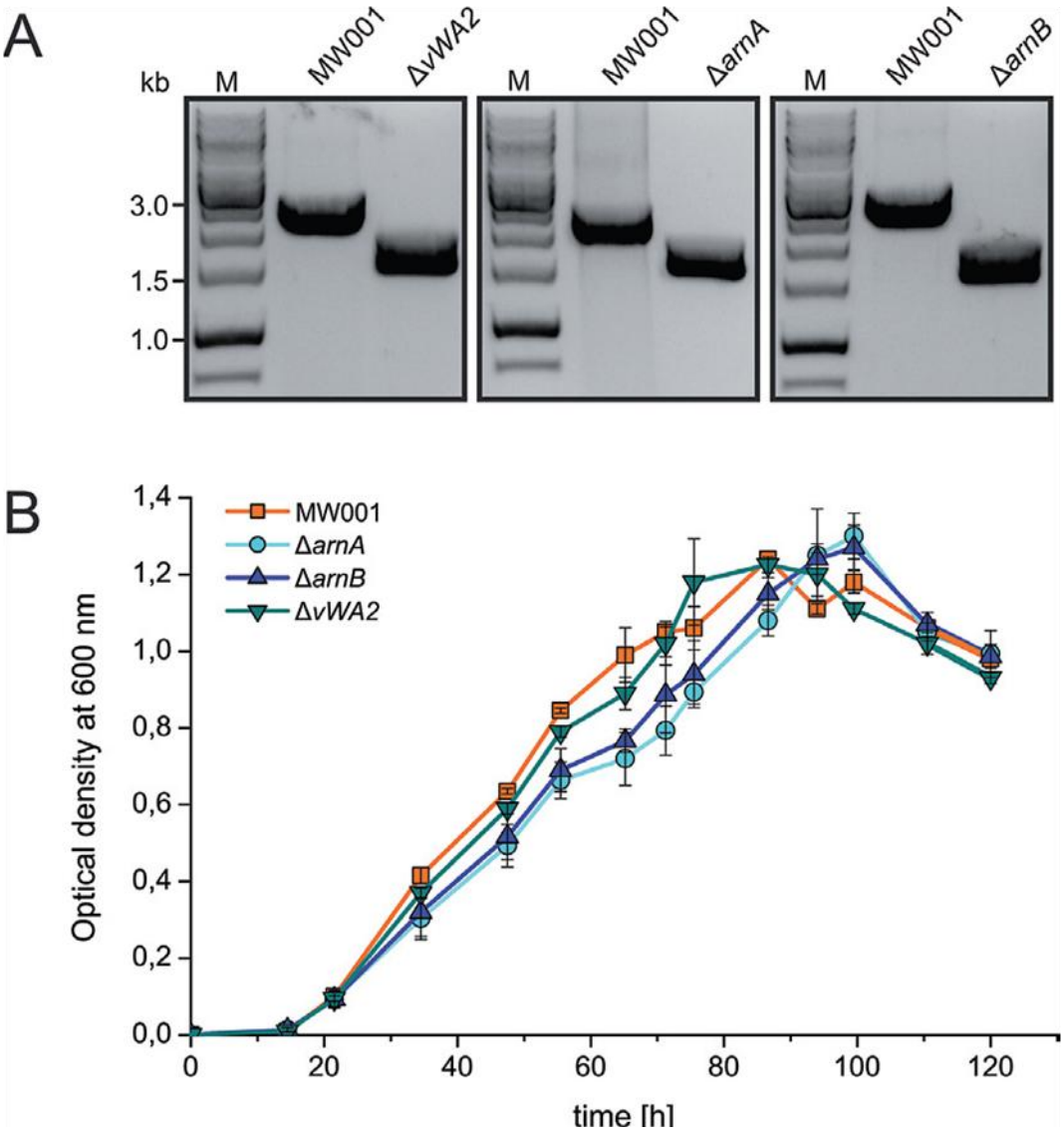



**Fig. 5** Confirmation of *vWA2*, *arnA* and *arnB* deletion mutants and determination of growth. **A**. Based on a single-cross-over event, deletion mutants of all three genes were constructed in the uracil auxotrophic background strain MW001. The PCR products of the mutants run faster than the wild-type products on agarose gel. M, marker. **B**. Growth curve of *ΔvWA2*, *ΔarnA* and *ΔarnB* in comparison with the wt strain MW001. Cells were grown on Brock medium supplemented with 0.1% NZ-amine, 0.2% sucrose and 10µg $ml^{-1}$ uracil at 76°C. The growth of all strains is comparable.

To verify the regulatory effect of ArnA and ArnB on archaella expression in *S. acidocaldarius* a motility assay was performed on semi-solid gelrite plates containing reduced amounts of the energy-source NZ-amine (0.005%) to induce starvation. Motility of *ΔarnA* and *ΔarnB* was compared with the motility of MW001 and *ΔaapF*. The latter strain lacks the aap pili which are involved in surface attachment and has been demonstrated to be hypermotile due to an overexpression of the archaellum operon (Henche *et al.*, 2012; Lassak *et al.*, 2012). After 5 days of incubation at 76°C, the *ΔarnA* and Δ*arnB* cells showed a comparable swimming radius like *ΔaapF*, whereas *ΔvWA2* and MW001 showed almost no motility after this incubation time (Fig. 7A). As described for the Western blot analysis, also in the motility plate assay the double-deletion strain

*ΔarnAΔarnB* showed the same phenotype as the single-deletion mutants (Fig. 7A). This hypermotile phenotype could be trans-complemented using a plasmid with the *arnAarnB* operon including the own promoter and terminator regions (Fig. S3).

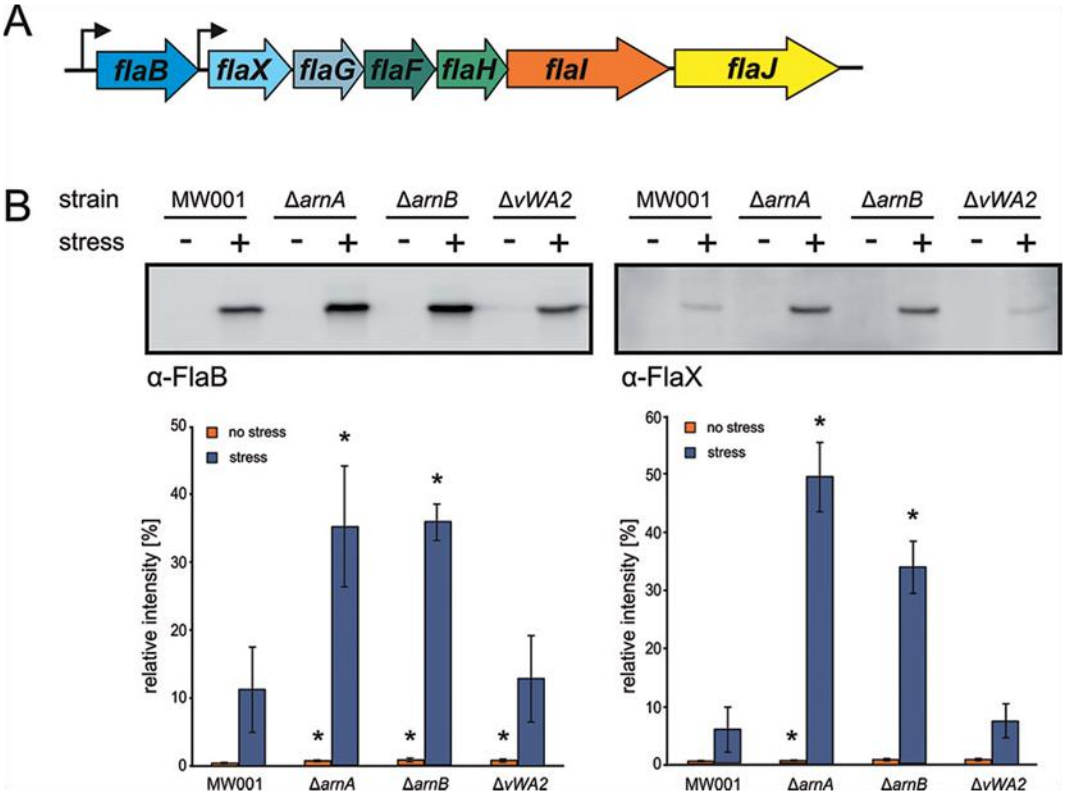



**Fig. 6** Expression levels of FlaB and FlaX in MW001, *ΔarnA, ΔarnB* and *ΔvWA2*. **A**. The archaellum is encoded in an operon consisting of seven genes and two indicated promoter regions in *S. acidocaldarius*. **B**. During tryptone-limiting growth conditions archaella expression can be specifically induced. FlaB and FlaX expression levels were detected by immunoblotting with specific antibodies before and after induction via tryptone starvation (top). Four independent stress experiments of the immunoblots were quantified using ImageJ (bottom). The statistical significance in comparison with the wild-type strain MW001 is indicated with a star (*$P$<0.05).

To further analyze the effect of the *arnA* deletion on motility in *S. acidocaldarius*, the swimming velocity of *ΔarnA* was determined and compared with the wild-type strain MW001 and the hypermotile strain *ΔaapF* (Lassak *et al.*, 2012) by thermomicroscopy. Upon tryptone starvation single *S. acidocaldarius* cells were tracked from the recorded movies via ImageJ to determine their swimming velocity. In comparison with the velocity of 30 μm $s^{-1}$ from MW001 and of 48 μm $s^{-1}$ for the hypermotile strain *ΔaapF*, *ΔarnA* swims with an intermediate velocity of 35 μm $s^{-1}$ (Fig. S4). Furthermore, not only the velocity, but also the number of swimming cells under inducing conditions was higher than in the wild type, with about 9% and 31% of swimming cells in MW001 and *ΔarnA* respectively.

Electron microscopy was used to further investigate the influence of ArnA on the production of surface structures in *S. acidocaldarius*. Consistent with the previous data, *ΔarnA* expressed a high level of archaella in tryptone starvation conditions (Fig. 7B). The *ΔarnB* strain did not show this

hyperarchaellation effect, but about six times more cells were archaellated. When using an optimized maltose inducible expression system for S. *acidocaldarius* (Berkner *et al.*, 2007), overproduction of ArnA resulted in hyper-piliation during normal growth conditions while hardly any archaella were expressed in nutrient-limited conditions (Fig. 7B).

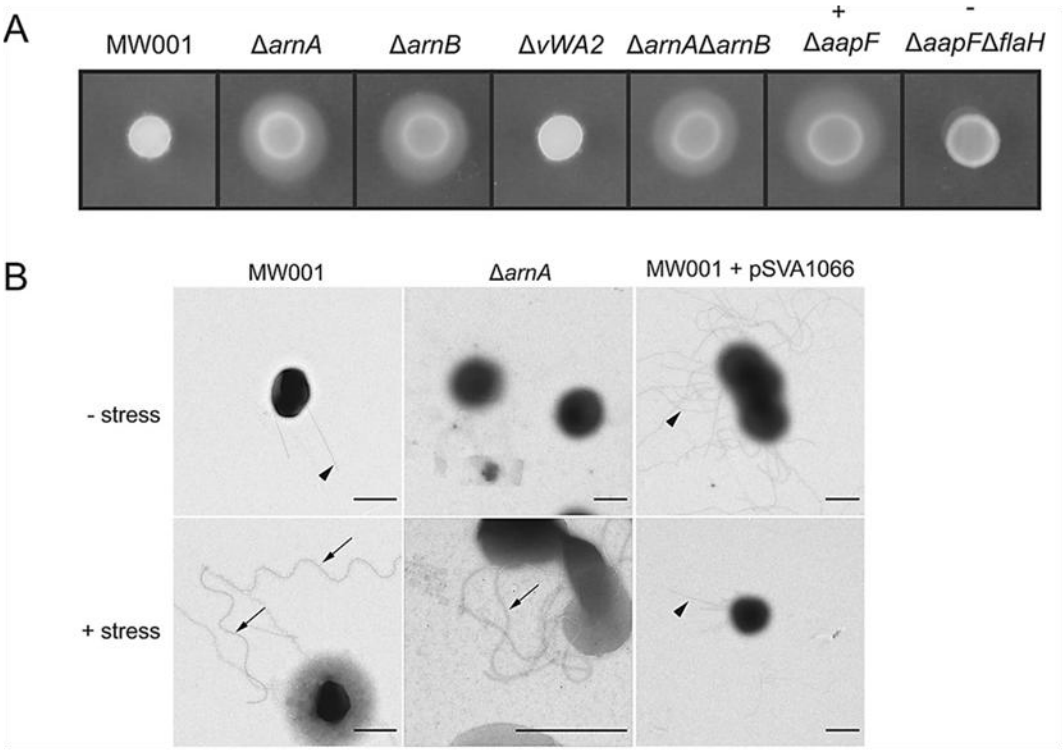



**Fig. 7** Motility assay with *S. acidocaldarius* MW001, *ΔarnA*, *ΔarnB*, *ΔvWA2* and the positive (*ΔaapF*) and negative (*ΔflaHΔaapF*) control and electron microscopy. **A**. Growing cultures of $OD_{600}$ ~ 1 were spotted on semi-solid gelrite plates with only 0.005% of NZ-amine and were incubated for 5 days at 76°C. **B**. Electron micrographs of MW001, *ΔarnA* and MW001 + pSVA1066 (overexpression plasmid for ArnA) during normal growth (- stress) and tryptone starvation (+ stress). Indication of archaella (arrows) and pili (arrowheads). Negative staining with uranyl acetate. Bars: 1µm.

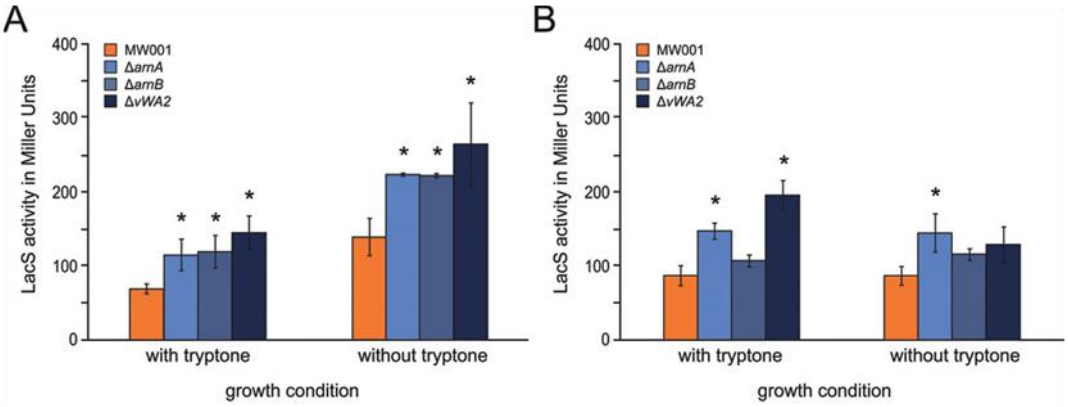



**Fig. 8** Promoter activity assay for both *fla* promoters. Reporter gene constructs with *flaB* (**A**) or *flaX* (**B**) promoter upstream of *lacS* were transformed into MW001, *ΔarnA*, *ΔarnB*, *ΔvWA2* and *Δppp*. The LacS activity was measured based on ONPG conversion and is described as Miller Units. The statistical significance in comparison with the wild-type strain MW001 is indicated with a star (*$P$<0.05).

**Archaella promoter activity assay**

To determine the influence of the *arn* cluster on the archaella promoters p*flaB* and p*flaX*, pSVA1600 and pSVA1601, were transformed into MW001, *ΔarnA, ΔarnB* and *Δ*vWA2 respectively. In pSVA1600 and pSVA1601, the maltose promoter of the reporter gene construct pCMal-LacS was replaced by p*flaB* or p*flaX* (Berkner *et al.,* 2010). The promoter activities driven by *flaB* and *flaX* promoter regions in the different deletion strains were then tested determining LacS activity as described before under optimal and tryptone starved growth conditions (Lassak *et al.,* 2012). In comparison with the wild type the *flab* promoter was significantly upregulated in *ΔarnA* and *ΔarnB* (Fig. 8A), whereas the *flaX* promoter was more pronounced upregulated in *ΔarnA* under non-inducing and inducing conditions (Fig. 8B). In contrast to the previous results also in *ΔvWA2* both promoters showed an upregulation in both conditions. In total, the influence of the gene deletion was not as high as expected, which leads to the speculation that regulation of the archaellum by ArnA and ArnB is not necessarily based on transcriptional regulation, but could also be based on protein–protein interaction. This observation is also supported by the fact that quantitative RT-PCR did not show higher *flaB* and *flaX* transcript levels in *ΔarnA* in comparison with MW001 under induced conditions (data not shown).

# DISCUSSION

Motility is a vital function for most single cellular organisms. In the crenarchaeon *S. acidocaldarius* motility is based on the archaellum (Jarrell and Albers, 2012), which is functionally analogous to the bacterial flagellum and structurally related to type IV pili of Gram-negative bacteria. The crenarchaeal archaellum is encoded in a genomic cluster comprising seven genes, which were shown to be controlled by two promoters in *S. acidocaldarius*, one upstream of the archaellin-encoding gene *flaB* and one upstream of the second gene *flaX* (Lassak *et al.,* 2012). In the exponential growth phase archaella gene expression can be specifically induced upon tryptone starvation conditions (Lassak *et al.,* 2012). This study provides first insights into the protein modules that underlie the regulation of archaella expression in *S. acidocaldarius*.

Interestingly, among the *Sulfolobales* the *arn* gene cluster genes encoding the FHA protein ArnA and the vWA protein ArnB always localize in each others direct vicinity. The presence of the *arn* gene cluster in *Picrophilus* is best explained by inferring a horizontal gene transfer event from a member of the *Sulfolobales*. Based on the results presented in the current study, it is unlikely that the *arn* genes are involved in cell division related activities, as has been proposed by Makarova *et al.,* (2010). Since the *P. torridus* genome lacks archaellum-encoding genes, we suggest that the *arn* genes are part of an alternative regulatory network in this organism.

The conserved clustering of the phosphatase-encoding gene within the *arn* genes in *Sulfolobus solfataricus* and the protein kinase gene in the *Desulfurococcales* suggest a possible involvement of phosphorylation events in modulating the function of ArnA and ArnB. *In vitro* studies revealed phosphorylation of ArnA and ArnB by the ePK Saci_1193 and specific phosphorylation of ArnB by the ePK Saci_1694. Since FHA domains are well-studied phosphopeptide binding modules, one can speculate if phosphorylation of ArnB is needed for interaction of ArnA with ArnB. However, co-purification revealed a strong interaction of ArnA and ArnB not only in the homologous, but also in the heterologous expression system. Therefore either ArnB can be phosphorylated in *E. coli* or ArnB does not necessarily need to be phosphorylated to interact with ArnA, which will be

subject to future studies. ArnA was phosphorylated *in vitro* by the ePK Saci_1193. Mutation of the conserved serine, which is involved in phosphopeptide binding in FHA domains, to alanine at position 146 in ArnA led to reduced phosphorylation levels. This suggests that more than one phosphorylation site is present on ArnA, since phosphorylation was not completely abolished. Unfortunately, we were so far unable to determine the phosphorylation sites on ArnA and ArnB by mass spectrometry. For *M. tuberculosis* the FHA protein Rv0020c could not be phosphorylated *in vitro* if the conserved Arg residue, responsible for phosphopeptide binding in the FHA domain, was mutated to an alanine (Grundner *et al.,* 2005). In contrast, the R132A mutation in the *S. acidocaldarius* ArnA was still phosphorylated, in line with the results for *S. tokodaii* (Wang *et al.,* 2010). This suggests that in the *Sulfolobales* FHA protein phosphorylation is not dependent on phosphopeptide interaction. Since vWA2 was not co-purified with ArnA the phosphorylation pattern of ArnB could lead to specificity in interaction of ArnA and ArnB.

For the first time a negative regulatory effect of ArnA and ArnB on archaella expression was shown *in vivo*. Deletion mutants of *arnA* and *arnB* revealed higher expression levels of FlaB and FlaX, hypermotility on plate, evoked by hyperarchaellation. Since the double-deletion mutant *ΔarnAΔarnB* did not show any additional phenotype than compared with the single mutants, ArnA and ArnB seem to regulate archaella expression at the same hierarchical level, which is in line with the observation that they do interact strongly. Interestingly, the vWA2 protein, which most likely emerged from a gene duplication event of *arnB*, showed no phenotype related to archaella expression in motility assays and protein level analysis, although both *flaB* and *flaX* promoter activities were induced in the *ΔvWA2* deletion strain. Promoter activity assays of *flaB* and *flaX* showed significant but only slightly increased levels of LacS activity in *ΔarnA* and *ΔarnB*. However, the effect is not as high as expected compared with the other *in vivo* assays. For the *S. tokodaii* FHA protein *in vitro* binding to the *flaX* promoter suggested a direct regulation of the archaellum on transcriptional level (Duan and He, 2011). Since the promoter activities were not as high as expected from the *in vivo* results in *S. acidocaldarius*, this might indicate that ArnA and ArnB do not regulate archaella expression on transcriptional level, but possibly on the protein–protein interaction level. The zinc finger domain in ArnA belongs to the RanBP type. Zinc finger domains of this type play a role in RanGDP binding. Therefore, ArnA might be also involved in regulatory processes based on protein interaction in the cell.

ArnA and ArnB could be dephosphorylated *in vitro* by the Ser/Thr phosphatase PPP. Loss of dephosphorylation of ArnA/ArnB could for example lead to a 'constitutive on' state of archaella expression. However, the loss of repression is only found under nutrient-limiting conditions implicating that another factor is needed that positively induces expression of the archaella operon under starvation conditions. Very recently such a factor was identified in *Sulfolobales* genomes (K. Lassak and S.V. Albers, unpublished) demonstrating that *S. acidocaldarius* employs a sophisticated regulatory network to regulate and adapt archaella expression to subtle changes in the environment. Furthermore, regulation of archaellum expression (for motility) and aap pili expression (for attachment) might be connected, since overexpression of ArnA lead to hyperpiliation. This hypothesis will be addressed in future studies.

In summary, this study gives first insights into the *in vivo* archaella regulatory processes via ArnA and ArnB and confirms the involvement of phosphorylation events in regulation of archaella expression in *S. acidocaldarius.*

# EXPERIMENTAL PROCEDURES

**Strains and growth conditions**

The uracil auxotrophic strain *S. acidocaldarius* MW001 (Wagner *et al.,* 2012) and all markerless deletion mutants were grown in Brock's basal medium (Brock *et al.,* 1972) at pH 3-3.5 and 76°C. Additionally, the medium was supplemented with 0.1% (w/v) tryptone or NZ-amine and 0.2% dextrin, sucrose or maltose.

For heterologous expression of the *S. acidocaldarius* genes, *E. coli* BL21 (DE3) RIL and Rosetta™(DE3)pLysS were used. Propagation of plasmids was conducted in *E. coli* DH5α cells.

**Expression of recombinant proteins in *E. coli***

Luria-Bertani medium (1 L) with 0.4% (w/v) glucose, 50 μg $ml^{-1}$ ampicillin and 30 μg $ml^{-1}$ chloramphenicol was inoculated with 1% of an overnight culture. Cells were grown at 37°C until they reached an OD600 of 0.5–0.6 and then induced with 0.5 mM IPTG (isopropyl β-D galactopyranoside). Subsequently the cultures were shifted to 16°C overnight to reduce inclusion body formation. The cells were harvested at 9000 *g* (Beckman Coulter Avanti J-26XP, rotor JLA 8.1), resuspended in lysis buffer (for His-tag purification 50 mM Tris pH 8, 150 mM KCl, 10 mM Imidazole; for Strep-tag purification 100 mM Tris pH 8, 150 mM NaCl), frozen in liquid nitrogen and stored at -80°C until further treatment.

**Purification of recombinant proteins in *E. coli***

Frozen cells were thawed on ice and lysed by sonication (40 min, intensity of 60% and interval of 1 min, Bandelin Sonopuls). Cell debris was removed by low spin centrifugation at 4500 *g* (Thermo Schientific Heraeus Multifuge 3SR+). Membranes were separated with ultracentrifugation at 236 000 *g* for 45 min (Beckman Coulter Optima MAX-XP, rotor MLA-55). To remove most *E. coli* proteins, a heat step was conducted with the soluble fraction at 70°C for 10 min, cooled down on ice and subsequently centrifuged at 236 000 *g* for 30 min. The supernatant was used either for His-tag purification or for Strep-tag purification. For additional purity of the proteins gel filtration was performed (ÄKTAPurifier, GE Healthcare).

**Transformation of plasmids into *S. acidocaldarius***

Preparation of competent cells, methylation of plasmids and transformation was basically performed as described by Wagner *et al.,* (2012). Methylated expression plasmids were transformed into MW001, recovered 30 min at 76°C in Brock medium supplemented with 0.1% NZ-amine and 0.2% sucrose, before plating them on gelrite plates supplemented with 0.1% NZ-amine and 0.2% sucrose.

**Expression and purification of proteins in *S. acidocaldarius***

Four hundred millilitres of Brock medium supplemented with 0.1% NZ-amine and 0.4% D-maltose at pH 3–3.5 was inoculated with 5-10 ml of a 2-day-old *S. acidocaldarius* culture MW001 harboring the respective expression plasmid. After reaching $OD_{600}$ of 0.7–0.8, cells were harvested by centrifugation at 9100 *g* (Beckman Coulter Avanti J-26XP, rotor JLA 10.5) and resuspended in

buffer A (50 mM Tris pH 8, 150 mM KCl, 10 mM imidazole and 1 mM PMSF). Cells were disrupted using sonication (20 min, intensity of 60% and interval 1 min, Bandelin Sonopuls) and cell debris was removed by 15 min centrifugation at 4600 rpm. Afterwards membranes were separated from cytoplasm using ultracentrifugation at 250 000 *g* for 45 min (Beckman Coulter Optima MAX-XP, rotor TLA-110). The soluble fraction was used for standard His-tag purification with His-Select Nickel Affinity Gel (Sigma). After binding of the tagged proteins, the column was washed once with 10 column volumes (CV) of buffer A, once with one CV of buffer B (buffer A with 20 mM imidazole) and eluted with three times one CV of buffer C (buffer A with 200 mM imidazole).

**Construction of in-frame deletion mutants**

The PCR product of about 600-800 bp up- and downstream of the gene of interest were fused via overlap PCR and cloned into the gene targeting plasmid pSVA406 (Wagner *et al.,* 2012). The resulting plasmids were methylated and transformed into *S. acidocaldarius* MW001. Deletion mutants were constructed as described in detail by Lassak *et al.,* (2012). In short, after transformation cells were grown on selective gelrite plates for the first and the second selection. Afterwards cultures were screened by colony PCR for the correct clones that were confirmed by sequencing.

**Functional and phylogenetic analysis of arn gene clusters and proteins**

Protein domain analysis was performed using the PFAM database (Punta *et al.,* 2011). The phylogenetic tree of vWA domain-containing proteins was constructed by multiple sequence alignment of archaeal vWA protein orthologues using MAFFT (v6.84b) (Katoh *et al.,* 2005) followed by phylogenetic inference using RAxML (Stamatakis *et al.,* 2005) and the LG model of protein evolution (Le *et al.,* 2008).

**Immunoblotting analysis after nutrient-limited growth conditions**

For Western blot analysis of FlaB and FlaX expression cells were tryptone starved. To that end, liquid cultures of *S. acidocaldarius* were grown in Brock medium supplemented with 0.1% tryptone and 0.2% sucrose till an $OD_{600}$ of 0.4–0.6. The cells were centrifuged at 2000 *g* and resuspended in fresh Brock medium without nitrogen and carbon source (stress culture) and with 0.1% tryptone and 0.2% sucrose (control culture). After 5 h of incubation at 76°C, the stressed cells were harvested to equal amounts of cells referring to the $OD_{600}$. Samples were separated on reducing SDS-PAGE and blotted on a PVDF membrane. The membrane was incubated with a 1:1000 dilution of polyclonal rabbit antibodies against FlaB and FlaX (Eurogentec). After incubation with the alkaline phosphatase coupled goat anti-rabbit secondary antibodies (Sigma), the chemiluminescence of the Western blot was detected with CDP-Star (Roche). Four independent experiments were performed and the relative signal intensity determined using ImageJ (http://rsb.info.nih.gov/ij/docs/menus/analyze.html#gels). The statistical significance of each strain compared with the wild-type MW001 was calculated with Origin 6.1 using independent *t*-test ($P<0.05$).

**Protein interaction study**

In the heterologous expression system [*E. coli* BL21(DE3)RIL] pSVA2216 was used for coexpression of His-tagged *arnA* in MCSI and Strep-tagged *arnB* in MCSII of the dual expression vector pETDuet-1 (Novagen). $ArnA^{Strep/His}$ and $ArnB^{Strep/His}$ were expressed homologously in *S. acidocaldarius* MW001 as described above using plasmids pSVA2221 and pSVA2222. Protein purification was conducted as mentioned above with His-Select Nickel Affinity Gel (Sigma). As controls heterologously in *E. coli* expressed $ArnA^{Strep}$ and $ArnB^{His}$ were used. The elution fractions and the control proteins were separated by SDS-PAGE and detected on Western blot with specific antibodies raised in rabbit (Agrisera AB, Sweden) against purified ArnA (1:5000 dilution) and ArnB (1:1000 dilution) heterologously expressed in *E. coli*.

**In vitro phosphorylation assay**

Purified proteins (0.5–3μM) were assayed in reaction buffer (25 mM MES pH 6.5, 150 mM KCl, 1 mM MnCl2) containing 32 nM [$\gamma$-$^{32}$P]-ATP (222 TBq mmol-1, Hartmann Analytics) and 0.8 mM ATP in a total volume of 15μl. Proteins were incubated for 30 min at 55°C. The reaction was stopped with the addition of 5X SDS loading dye. The samples were separated by SDS-PAGE and exposed to a Storage Phosphor Screen (Molecular Dynamics) overnight. Images were scanned with Storm 840 scanner.

**Motility assay on semi-solid gelrite plates**

Strains were grown till $OD_{600}$ of about 0.6-1 and the same amount of cells calculated on $OD_{600}$ were spotted on the semi-solid gelrite plate. The plates containing only 0.15% (w/v) of gelrite were prepared as described before (Lassak *et al.*, 2012), supplemented with 0.005% (w/v) tryptone or NZ-amine, 0.2% (w/v) dextrin or sucrose and 10μg $ml^{-1}$ Uracil in case of the deletion mutants. The plates were incubated for 5 days at 76°C in a metal box (Koerdt *et al.*, 2010) with water at the bottom to avoid drying of the plates.

**Transmission electron microscopy (TEM)**

The same material and the same treatment of cells were used as described in Lassak *et al.*, (2012). In short, cells were chemically fixed with 2.5% glutaraldehyde and applied to glow-discharged Carbon-coated 200-mesh copper grids (Plano, Germany). After washing twice with double-distilled water, negative staining with 2% uranyl acetate was conducted for 20 s. Grids were analysed on a JEOL 2100 TEM (JEOL, Tokyo, Japan), equipped with a LaB6 cathode and a fast-scan 2k X 2k camera F214 (TVIPS, Germany) and operated at 80 kV. The surface appendages were measured in diameter to assign them as pili or archaella.

**Thermomicroscopy**

For comparison of the swimming velocities of the wild-type strain *S. acidocaldarius* MW001 to the deletion mutant *ΔarnA* the latter was treated and analysed as described by Lassak *et al.*, (2012). About 300 cells of each strain were counted to compare the number of swimming cells.

**Promoter activity assay**

Promoter activity assays for *flaB* and f*laX* promoters were performed basically as described in Lassak *et al.,* (2012). Translational promoter fusions were cloned into pCMalLacS (Berkner *et al.,* 2010) to exchange the maltose promoter. These plasmids (pSVA1600 and pSVA1601) in addition to the promoterless control plasmid (pSVA1614) were transformed into MW001, *ΔvWA2, ΔarnA* and *ΔarnB.* The color reaction of cleaved o-nitrophenyl-β-D-galactopyranosid (ONPG) by LacS was detected at 410 nm and Miller units were calculated from the assay triplicates. The statistical significance of each strain compared with the wild-type MW001 was calculated with Origin 6.1 using independent *t*-test ($P<0.05$).

**Strains and plasmids**

Strain and plasmid lists are shown in *Supporting information* (Tables S1 and S2).

## ACKNOWLEDGEMENTS

We thank Reinhard Wirth (University of Regensburg, Germany) for access to the thermomicroscope. J.R. was supported by the BMBF project 'SulfoSYS' (0315004C), S.-V.A. by intramural funds of the Max Planck Society and N.Y. by the Chinese Scholar Council. K.L. received funding from the International Max Planck Research School for Environmental, Cellular and Molecular Microbiology.

# 6

# SUMMARY AND CONCLUSIONS

## INTRODUCTION

Archaea, as a third domain of life (Woese *et al.,* 1977), are ubiquitous and thrive in diverse ecological niches. They represent an interesting group of organisms both with respect to evolution, adaptation and stress response mechanisms in particularly associated with life under extreme environments. Archaea share characteristics with bacteria, which goes beyond their morphology. This concerns various metabolic traits and life styles. The basal cellular functions of archaea, however, show more similarity to eukaryotes including the replication, transcription and translation machinery. They also show some unique features of their carbon- and nitrogen metabolism. *Sulfolobales* are considered as model organisms for the phylum Crenarchaeota thriving mainly in volcanic springs at temperatures around 80˚C and low acidic pH (pH 2-3). They are the only representatives and genetically tractable members of the Crenarchaeota. Since different members of the *Sulfolobales* were isolated and their genomes have been sequenced and annotated, comparative analysis of the metabolic traits now provides a deeper understanding of their functioning. These developments have also led to the construction of genetic tools that allow targeted gene deletions and protein expression. In particular *S. acidocaldarius*, which is a hyperthermoacidiphilic organism discovered in Yellowstone National Park (Brock *et al.,* 1972), is more readily assessable for this gene deletion methodology making it a prime candidate for genetic studies (Wagner *et al.,* 2009) and for the work described in this thesis.

## ARCHAEAL CELL DIVISION

A peculiar feature of *Sulfolobalen* is their ability to release membrane vesicles from their cell surface. These vesicles are coated with S-layer protein and their membrane lipid composition resembles that of the cytoplasmic membrane. However, these membrane vesicles have a protein composition that differs from the cytoplasmic membrane, and some proteins appear particular abundant such as two proteins that are homologous to ESCRT-III that is part of the eukaryotic endosomal sorting complex required for transport-III (ESCRT-III) system, and a fragment of a Vps4-like protein that in eukaryotes is also part of the aforementioned sorting complex. Although their exact origin is unknown, the surface derived membrane vesicles have been associated with cell division since the ESCRT-III homologs appear essential for this process in the majority of Crenarchaeota and likely all Thaumarchaeota. This cell division system (Cdv) consists of three highly conserved genes, *cdvA*, *cdvB* and *cdvC* that are organized in an operon. This system shows homology to the eukaryotic ‘‘endosomal sorting complex required for transport (ESCRT)’’ machinery. CdvB is homologous to Vps24, which presumably functions as a core component of the ESCRT-III system. Next to the *cdv* operon, most Crenarcheota contain additional CdvB paralogs, in *S. acidocaldarius* these are termed CdvB1-3, that are differentially expressed under conditions of cell cycle, viral infection and others. These three additional CdvB homologs proteins interact with themselves and each other but unlike CdvB they do not interact with CdvC. Since their exact roles are still unclear, we employed a gene deletion strategy to assess their function in cell division (**Chapter 2**). Except for the *cdvB* gene, the *cdvB1-3* genes could be deleted suggesting a redundant function although the resultant strains varied in the extent of their growth defect. In particular the *ΔcdvB3* strain showed a severe growth defect with the formation of enlarged cells and a defect in DNA segregation.

Without CdvB3, cells with duplicated genomes may not divide on time, and continue to grow in size even though at a lower rate, but eventually they divide. The phenotype is accompanied

with a lower expression of the core *cdvABC* genes that likely contributes to a more pleiotropic growth defect. Although the lower expression of these genes may be responsible for the reduced growth rate which is in particular strong with plate-grown cells, the restoration of their expression during the mid-log phase in liquid cultures of the *ΔcdvB3* strain suggests that CdvB3 fulfills a coordinating role rather than an essential function.

In the wild-type strain, cell division seems to be associated with a mid-cell localization of a ring-like structure of the CdvA protein that may be bound to the membrane and possibly also interacts with DNA. This membrane bound ring then recruits CdvB to form similar ring-like structure. As the ring constricts, possibly at the expense of ATP hydrolysis by CdvC, the cell divides into the two daughter cells. A prerequisite to nucleoid segregation and recruitment of CdvB is the assembly of CdvA. In this regard, due to the absence of CdvB3 in the mutant strain, the CdvA protein seems to assemble in a spot-like structure rather than in a band or ring-like structure. Likely, this structure is defective in the recruitment of CdvB, hence the low CdvB fluorescence in these cells. Also membrane invagination does not seem to occur in the enlarged *ΔcdvB3* mutant cells. Therefore, the aberrant cellular localization of CdvA and CdvB in the *ΔcdvB3* mutant cells further suggests a coordinating function of CdvB3 in cell division.

Deletion of the other two CdvB paralogs (CdvB1 and CdvB2, respectively) did not show a strong phenotype related to cell division. Interestingly, they are present in the secreted membrane vesicles in *S. acidocaldarius* while in previous studies they have been implicated in virus release. Our data indicate that CdvB1 and CdvB2 do not fulfill a critical role in cell division, nor is it clear if they are involved in vesicle formation. Future studies should address if cell division and vesicles formation are truly interlinked processes, or whether vesicle formation is a primordial phenomenon associated with viral biogenesis or some other form of secretion. In particular it will be of interest to determine if the *ΔcdvB1* and *ΔcdvB2* cells still form membrane vesicles. Technically, this is a demanding task as even in the wild-type strain, the yield of vesicles during exponential growth is low whereas in the late log and stationary phase, cell lysis interferes with the analysis.

## ABC-MDR TRANSPORTER

In *Sulfolobalen*, ABC-type transporters appear in particular important for their survival strategy in the oligotrophic extreme environments. Such hostile environments likely require further mechanisms for cellular protection, for instance, to provide for protection against natural occurring or anthropogenic toxic compounds. ABC-MDR transporters appear in particular effective in cellular protection as the coupling to ATP hydrolysis allows an efficient secretion against a large concentration gradient. However, hardly any studies have been performed on this type of transporter in archaea despite the fact that many these microorganisms thrive in hostile environments where there is a need for cellular protection against toxic molecules. So far, only a protein of the small multidrug resistance (SMR) family of transporter proteins, Hsmr, was identified in *Halobacterium salinarum*, and indirect evidence hints at a functional role of an ABC-MDR transporter in *Haloferax volcanii*. Bioinformatics analysis revealed the presence of both primary and secondary putative multidrug transporters in *S. acidocaldarius*. Nearly all of the annotated ABC-MDR like transports exhibit a domain organization which is typical for an ABC type uptake system as they cluster with a gene encoding a solute binding protein. However, Saci_2123 (here termed *Sulfolobus* multidrug resistance, Smr1), is a so-called "half-transporter" consisting of a membrane domain fused at the C-terminus to an ATP binding domain (**Chapter 3**).

This protein is conserved in related *Sulfolobales*. Smr1 indeed exhibits homology to well-characterized bacterial and mammalian MDR transporters. They share not only a common core-architecture, but are also equipped with common features, such as the ability to recognize and extrude diverse structurally and functionally dissimilar toxic substances.

This *smr1* gene is upregulated when cells are exposed to drugs, and a deletion mutant of *smr1* was found to be more vulnerable to a series of toxic compounds compared to the wild type strain. The drug sensitivity could be restored, and was even slightly higher than the wild-type strain when Smr1 was overexpressed using a plasmid-based system, suggesting that the observed drug resistance is indeed due to the expression of *smr1* and not associated with polar effects of the gene deletion on the expression of neighboring genes. Therefore, the Saci_2123 protein represents the first identified and initial characterized ABC multidrug efflux pump from Archaea for which a role in drug resistance could be demonstrated experimentally. It will be of interest to examine its role in the natural environment such as acidic high temperature volcanic springs. Smr1 is, however, significantly longer than the bacterial ABC-MDR-like transporters because of the presence of a DUF1854 domain at the C-terminus. This is a functionally uncharacterized domain that may be involved in ligand binding and so far has only been found in uncharacterized proteins. The functional role of this domain is unknown and it would be of interest to examine the growth phenotype of cells in which only this C-terminal extension of Smr1 is removed. In this respect, on regular Brock's medium, the *Δsmr1* strain has no obvious phenotype and therefore it will be of interest to examine such cells under chemolithotrophic growth conditions where sulfide is oxidized to sulfate. Smr1 may also be used as a selection tool in genetic studies. The development of a genetic system in *Sulfolobus* so far depends on auxotrophic markers. Screening for small toxic molecules for which Smr1 provides protection could possibly lead to the development of new selection markers.

## IRON ABC-BINDING PROTEIN

Iron is the most abundant metal ion in cells and required for multiple metabolic functions. It is usually not freely available in the environment but in complex with other molecules or as a non-soluble salt and present in the oxidized form. In most iron transport systems studied so far, uptake requires an ABC transporter as well as a periplasmic-binding protein with high specific substrate affinity. Little information is available on iron transport in Archaea. So far only in the halophilic *Halobacterium* ssp., siderophore production and iron uptake were studied in detail (Dave *et al.,* 2005; Hubmacher *et al.,* 2007). For the hyperthermoacidophilic Crenarchaeota, the question on the mechanism of iron transport is in particular of interest. Because of the acidic environment in which they thrive, ferric ions are much more soluble possibly avoiding the need for siderophores. Indeed, siderophores could not be detected in the culture supernatant of *S. acidocaldarius* consistent with the notion that genes specifying the production of known siderophores are absent from the genome. Likewise, two xenosiderophores from different siderophore-classes did not promote growth or uptake (**Chapter 4**). Uptake of $Fe^{3+}$ shows typically Michalis-Mentenkinetics with a $K_m$ value in the order of 4.68 μM. Interestingly, when cells were grown in Brock's medium, the uptake of $^{59}Fe$ was almost 2-fold higher than with cells grown under iron limiting condition, indicating that iron-depletion does not stimulate the uptake unlike what is typically found in micro-organisms where iron-limitation stimulates the expression of a range of high affinity iron uptake systems. Consistent with studies in other acidophiles

microorganisms, only a small number of putative iron transporter specifying genes are found in the *S. acidocaldarius* genome. The bioinformatics analysis revealed three potential iron binding proteins of which only one seems to cluster in the chromosome with the genes encoding an ABC transporter. Two of the putative iron binding proteins, one which co-localizes with the genes encoding an ABC transporter are upregulated when cells were grown in iron-limited medium. Deletion of the binding protein genes caused a growth defect both in iron-defect and rich medium as compare with their parent strain, and also affected the uptake of iron. In the individual gene deletions, compensatory gene expression levels appeared to occur possibly counteracting the defect introduced by the single deletions. However, double and triple deletions did not strengthen growth defect, suggesting that besides these binding protein dependent mechanisms, other transport mechanisms must prevail in *S. acidocaldarius* for the uptake of iron. This could for instance involve members of the NRAMP family but these were not targeted experimentally. Future studies should assess the role of additional transport mechanisms in the uptake of iron and delete the complete ABC transporter that might be involved in the uptake of iron.

## REGULATION OF ARCHAELLA EXPRESSION

The ability of microorganisms to sense and respond to sudden changes in their environment often involves regulatory systems comprising reversible protein phosphorylation. In Archaea the archaellum (former: archaeal flagellum) that is functionally analogous to the bacterial flagellum is used for motility, which is a vital function for most single cellular organisms. The archaellum is encoded in a genomic cluster genes that were controlled by two promoters: *flab* and *flax* (Lassak *et al.,* 2012). In contrast with Euryarchaeota, regulation of archaellum-mediated motility remains poorly understood in crenarchaeal organisms like *Sulfolobus* species. Interesting, archaella expression and assembly is induced under trypton limiting growth conditions in *S. acidocaldarius*. The *arn* gene cluster genes encode the Forkhead-associated FHA protein ArnA and the von Willebrand factor vWA protein ArnB that always co-localize as genes. These two proteins are likely interacting proteins, and remarkably both were found as constituents of the surface-derived membrane vesicles. This raised the question if they are involved in an alternative regulatory network, and how they are linked to vesicle formation. Therefore the genes encoding ArnA and ArnB were targeted for gene deletion. Remarkably, the gene deletion mutants were found to show a defect in biofilm formation possibly linking them to motility and/or surface adherence, while a role in membrane vesicle formation could experimentally not be demonstrated. These basic observations fueled further studies on the regulation of archaella expression in *S. acidocaldarius*.

*In vitro* both ArnA and ArnB proteins are phosphorylated by specific protein kinases and they were found to co-purify indicating a strong interaction between these proteins. Phosphorylation was not completely abolished by mutating one of the conserved phosphopeptide binding FHA domains in ArnA, suggesting that there is more than one phosphorylation site in ArnA. Unfortunately, we were unable to identify the other phosphorylation sites on ArnA and ArnB. Either ArnB can be phosphorylated by an endogenous system in *E. coli* that was used to express these proteins or the unphosphorylated ArnB interacts with ArnA.

Phenotypic analyses revealed a negative regulatory effect of ArnA and ArnB on archaella expression. Deletion mutants of *arnA* and *arnB* revealed higher expression levels of FlaB and FlaX, hypermotility on plate, evoked by hyperarchaellation, and seem to they regulate archaella expression at the same hierarchical level, which is in line with the observation that they interact

strongly. These results represent the first step in understanding the network that underlies the regulation of cellular motility in Crenarchaeota and emphasize the importance of protein phosphorylation in the regulation of cellular processes in the Archaea. The role of ArnA and ArnB in membrane vesicle formation, if any, however, remains to be elucidated.

# 7

# SAMENVATTING EN CONCLUSIES

## INTRODUCTIE

Archaea, het derde domein van het leven (Woese*et al.,* 1977), zijn alom vertegenwoordigdengedijenin verschillend ecologischeniches. Ze vertegenwoordigen een interessante groep van organismen als men kijkt naar evolutie, adaptatie en stressrespons mechanismen in het bijzonder in combinatie met leven onder extreme omstandigheden. Archaea delen eigenschappen met bacteriën dat verder gaat dan het morfologische aspect. Dit betreft verschillende metabole eigenschappen en levensstijlen. Uit de basale cellulaire functies van Archaea blijkt dat ze meer gemeen hebben met eukaryoten wat betreft vermenigvuldiging, transcriptie en translatie. Ze laten ook enkele unieke kenmerken in hun koolstof- en stikstofmetabolisme zien. *Sulfolobales* worden als model organismen beschouwd omdat de phylum Crenarchaeota groeit in vulkanische geisers met een temperatuur van rond de 80˚C en pH van 2 a 3. Ze zijn de enige vertegenwoordigers en genetisch handelbare leden van Crenarchaeota familie. Omdat verschillende leden van de *Sulfolobales* familie zijn geïsoleerd en diens genoom gesequenced en geannoteerd is, geeft een vergelijkende analyse van de metabole eigenschappen een dieper inzicht in hun functioneren. Deze ontwikkelingen hebben er toe geleid datmet het gebruik van genetische gereedschappen genendeleties en eiwitexpressies geïnitieerd kunnen worden. In het bijzonder *S. acidocaldarius,* een hyperthermoacidofiel organisme ontdekt in Yellowstone National Park (Brock *et al.,* 1972), welke makkelijker doelwit is voor de genendeletie methode waardoor het een uitstekende kandidaat is voor genetische studies (Wagner *et al.,* 2009) en voor het werk dat in deze thesis behandeld wordt.

## CELDELING IN ARCHAEA

Een bijzondere eigenschap van de *Sulfolobalen* is hun vermogen om lipide vesikels van het membraan af te stoten. Deze vesikels, of blaasjes, hebben een eiwit-laag aan de buitenkant genaamd de S-laag, terwijl de lipide samenstelling van het binnen-membraan van deze blaasjes overeenkomt met het cytoplasmatisch membraan van *Sulfolobalen*. De eiwitsamenstelling van het binnenmembraan verschilt echter van het cytoplasmatisch membraan, waar een bijzonder grote hoeveelheid van twee eiwitten wordt gevonden. De aminozuurvolgorde het eerste eiwit lijkt op het ESCRT-III complex dat ook onderdeel is van het eukaryote endosomale sorteer-complex dat nodig is voor het transport-III systeem, terwijl het tweede eiwit een homoloog is van het Vps4-eiwit dat onderdeel is van hetzelfde transport-III complex. Hoewelde exacte herkomst van deze twee eiwitten onbekend is worden de van membranen afgeleide vesikels geassocieerd met celdeling omdat de ESCRT-III eiwitten een essentiële rol in dit proces blijken te hebben in veel Crenarchaeota en in bijna alle Thaumarchaeota. Dit celdelingssysteem (Cdv, van Eng. cell division) bestaat uit drie genen met een hoge overeenkomst, *cdvA*, *cdvB* en *cdvC*, allen onderdeel van één operon. Het Cdv-systeem komt overeen met het eukaryote ESCRT complex (van Eng. endosomal sorting complex required for transport). CdvB is homoloog aan Vps24, wat vermoedelijk het kern-onderdeel is van het ESCRT-III systeem. Naast het *cdv*-operon hebben de meeste Crenarchaeota extra CdvB paralogen, die in *S. acidocaldarius* CdvB1-3 genoemd worden en die op verschillende momenten in de celcyclus, en ook tijdens virale infectie, tot expressie gebracht worden. Deze drie aan CdvB paraloge eiwitten gaan een interactie met elkaar aan, maar niet met CdvC, iets watde oorspronkelijke CdvB wel doet.

Omdat de exacte functie van CdvB onduidelijk is hebben wij door middel van gen-deleties

geprobeerd de functie tijdens de celdeling te onderzoeken (zie **Hoofdstuk 2**). Met uitzondering van *cdvB* konden *cdvB1-3* allemaal uitgeschakeld worden – dit suggereert een overtollige (of dubbele) rol, hoewel de *cdvB1-3*-negatieve stammen wel een groeiafwijking lieten zien. De *ΔcdvB3*-stam had een bijzonder ernstige groeiafwijking, waarbij abnormaal grote cellen ontstonden met een slecht werkende scheiding van DNA.

Zonder CdvB3 kunnen cellen die hun genoom al gekopieerd hebben niet op tijd delen, en groeien daarom langzaam door totdat ze alsnog aan de celdeling beginnen. Dit fenotype gaat samen met een lagere expressie van de *cdvABC* genendie op hun beurt ook weer bijdragen aan een ingewikkelder groei-defect. Hoewel de lagere expressie van de *cdvABC*-genen verantwoordelijk kan zijn voor de verminderde groeisnelheid, wat bijzonder duidelijk is wanneer culturen op platen groeien, suggereert een herstel van groei van de *ΔcdvB3*-stam tijdens de mid-logarithmische fase in vloeibare culturen dat CdvB3 eerder een coördinerende rol heeft dan dat het een essentiële functie heeft.

In niet-gemuteerde stammen lijkt celdeling geassocieerd met een ring-structuur van CdvA in het midden van de cel. Mogelijk bindt CdvA kan hierbij aan het membraan en kan dan tegelijk een interactie aangaan met het DNA. Deze membraangebonden ring hecht dan CdvB die dan een daarop lijkende ring-structuur vormt. Als de ring samentrekt, mogelijk als gevolg van ATP-hydrolyse door CdvC, deelt de cel in twee dochtercellen. Een vereiste voor segregatie van de nucleoide en het hechten van CdvB aan CdvA is een correcte plaatsing van CdvA. In dit opzicht, door de afwezigheid van CdvB3 in de deletie-mutant wordt CdvA in een puntachtig patroon geplaatst in plaats van in een ring. Waarschijnlijk lijdt deze mis-plaatsing van CdvA tot verkeerde deling en een verkeerde binding met CdvB. In de *ΔcdvB3* stam vindt ook de samentrekking van het membraan niet plaats. Daarom suggereert een afwijkend lokalisatie-patroon van CdvA en CdvB in de *ΔcdvB3* mutant een coördinerende rol voor CdvB3 in de celdeling.

Het verwijderen van de twee andere CdvB paralogen (CdvB1 en CdvB2) leidde niet tot een duidelijk fenotype zien dat gerelateerd was aan celdeling. Interessant is datbeide eiwitten aanwezig zijn in membraan-vesikels van *S. acidocaldarius*, en in eerdere studies ook betrokken waren bij het afstoten van virusdeeltjes. Onze resultaten laten zien dat CdvB1 en CdvB2 geen sleutelrol spelen bij de celdeling, en ook is het niet duidelijk of ze betrokken zijn bij het maken van vesikels. Toekomstig onderzoek zou moeten uitwijzen of celdeling en vesikulatie met elkaar verbonden zijn, of dat vesikels geassocieerd moeten worden met het maken van virusdeeltjes of een andere vorm van secretie. Het is in het bijzonder interessant te onderzoeken of *ΔcdvB1* en *ΔcdvB2* stammen nog in staat zijn om membraan vesikels te maken. Dit is echter een veeleisende bezigheid omdat vesikels een lage opbrengst hebben als cellen exponentieel groeien, terwijl bij stationaire cellen de analyse van vesikels gehinderd wordt door cel-afbraak.

## ABC-MDR TRANSPORTER

ABC-transporters vervullen een belangrijke rol in de overlevingsstrategie van Sulfolobalen in extreme omstandigheden. Voor het overleven in zulke omstandigheden zijn vermoedelijk meerdere mechanismes nodig om de cel te beschermen, zoals tegen dodelijke antropogene stoffen. ABC-MDR transporters blijken bijzonder effectief in de bescherming van de cel omdat de koppeling met ATP-hydrolyse een efficiënte uitstoot tegen een grote concentratiegradiënt mogelijk maakt. Helaas is er weinig onderzoek gedaan naar dit type transporter in archaea, ondanks het feit dat deze micro-organismen goed groeien in een extreem milieu waar er behoefte is aan

bescherming tegen toxische stoffen van buitenaf. Tot nu toe is alleen een kleine multidrug resistente (SMR, Eng. small multidrug resistance) transporter genaamd HsmR gevonden in *Halobacterium salinarum*, en is er indirect bewijs voor het bestaan van een ABC-MDR transporter in *Haloferax volcanii*. Bioinformatische analyse suggereert de aanwezigheid van primaire en secondaire multidrug transporters in *S. acidocaldarius*. Bijna alle geannoteerde ABC-MDR-achtige transporters laten een domein-organisatie zien dat duidt op de aanwezigheid van een ABC-opname systeem. Van deze is Saci_2123 (vanaf nu genoemd Smr1, vanuit Eng. *Sulfolobus* multidrug resistance) een zogenaamde "halve transporter", bestaande uit een membraan-domein dat gefuseerd is met aan ATP-bindend domein (**Hoofdstuk 3**). Smr1 is geconserveerd gebleven in gerelateerde *Sulfolobales* stammen, en is zelfs homoloog aan de beter bestudeerde bacteriële en zoogdier MDR-transporters. Niet alleen de kern-architectuur komt overeen,ze hebben ook andere overeenkomsten, zoals de mogelijkheid om structureel verschillende stoffen uit de cel te verdrijven.

De expressie van het *smr1*-gen wordt verhoogd als cellen blootgesteld zijn aan giftige stoffen, en deletie van het *smr1*-gen leidde tot een verhoogde gevoeligheid voor deze stoffen. Deze verhoogde gevoeligheid kon worden gereguleerd en zelfs worden teruggebracht tot niveaus onder die van niet-gemuteerde stammen door overexpressie van *smr1* op een plasmide. Dit suggereert dat de gevonden resistentie komt door de expressie van *smr1* en niet van neveneffecten die geassocieerd kunnen zijn met gen-deletie en de expressie van naburige genen. Saci_2123 is daarom de eerst gevonden en gekarakteriseerde ABC multidrug eflux pomp in Archaea waarvoor een functie experimenteel kon worden aangetoond.

Het is van belang om de rol van deze transporter in zijn natuurlijke omgeving te onderzoeken, zoals een omgeving met hoge zuurgraad en hoge temperaturen, bijvoorbeeld in vulkanische bronnen. Smr1 is echter veel langer dan de bacteriële ABC-MDR transporters door de aanwezigheid van een DUF1854 domein aan de C-terminus. Dit domein, waarvan de functie niet bekend is, maar dat betrokken kan zijn bij de binding van liganden, is alleen nog maar gevonden in het DNA van niet eerder onderzochte eiwitten. De functie van dit domein is niet bekend en het is van belang om het fenotype van cellen te bepalen waarbij de C-terminale extensie van Smr1 is verwijderd. Echter wanneer cellen groeien op gewoon Brock's medium, heeft de *Δsmr1*-stam geen duidelijk fenotype en het is daarom belangrijk om ook cellen te onderzoeken onder chemilithotrofe omstandigheden waarbij sulfiden worden geoxideerd tot sulfaten. Smr1 zou ook ingezet kunnen worden als een selectie-tool voor genetische studies. De ontwikkeling van een genetisch systeem dat gebruikt kan worden in *Sulfolobus* is vooralsnog afhankelijk van auxotrofe selectiemethoden. Het screenen van kleine giftige stoffen waartegen Smr1 bescherming biedt kan mogelijk ook worden gebruikt voor de ontwikkeling van nieuwe genetische selectiemethoden.

## IJZER-BINDEND ABC-EIWIT

Het meest voorkomende metaalion in cellen is ijzer, wat nodig is voor verscheidene metabolische functies. Gewoonlijk is ijzer niet gemakkelijk te verkrijgen uit de omgeving, omdat het vaak bestaat in een complex met andere moleculen of als een niet-oplosbaar zout in een geoxideerde toestand. De tot nu toe onderzochte ijzer-opname systemen bestaan uit ABC-transporters met een periplasmatisch bindings-eiwit met een hoge affiniteit voor ijzer. In Archaea is vrij weinig bekend over ijzer transport, alleen is in de halofiele *Halobacterium*-stam de productie van sideroforen en ijzer-opname onderzocht (Dave *et al.,* 2005; Hubmacher *et al.,* 2007). Voor de

hyperthermoacidofiele Crenarchaeota zijn vragen rondom ijzer transport mechanismen van bijzonder belang, omdat in de zure omgeving waarin ze leven ijzer-ionen veel oplosbaarder zijn waardoor sideroforen niet meer nodig zijn. Dat verklaard waarom er nooit sideroforen zijn gevonden in media van *S. acidocaldarius* en er geen genen voor het maken van sideroforen zijn gevonden in het genoom. Het verklaard ook waarom het toevoegen van twee xenosideroforen van verschillende klassen niet tot snellere groei of meer opname van ijzer leidde (**Hoofdstuk 4**).

Opname van $Fe^{3+}$ gebeurt met een typische Michaelis-Menten kinetiek met een $K_m$-waarde van ordergrootte 4.68 μM. Interessant is dat in cellen die groeien op Brock's medium de opname van $^{59}Fe$ bijna tweevoudig was dan cellen die groeiden onder ijzer-gelimiteerde omstandigheden, wat betekent dat ijzer-depletie de ijzer-opname niet stimuleert, iets wat wèl vakergezien is in andere micro-organismen (gewoonlijk lijdt een ijzertekort tot hogere expressie van hoge-affiniteit ijzer-opname systemen). Wat overeenkomt met andere acidofiele micro-organismen is het kleine aantal ijzer-transport genen in het genoom van *S. acidocaldarius*. Een bioinformatische analyse resulteerde in drie potentiële ijzer-bindende eiwitten waarvan er slechts één geclusterd is met een ABC-transporter gen. Twee van deze ijzer-bindende eiwitten, waaronder die geclusterd met het ABC-transport gen, worden wèlopgereguleerd wanneer cellen groeien in een ijzer-limiterend medium. Verwijdering van deze eiwitten leidt tot een groei-defect in nutriënt-rijk medium vergeleken met de niet-gemuteerde stam, en daarbij werd ook ijzer-opname verstoord. In de individuele deletie-stammen was aanwijzing voor genexpressie met een compenserend effect dat mogelijk het effect van de deletie tegengaat. Echter, dubbele en driedubbele deleties versterkten het groei-defect niet, wat suggereert dat er nog een ander ijzer-transport mechanisme bestaat in *S. acidocaldarius*. Dat zou bijvoorbeeld een lid van de NRAMP familie kunnen zijn, maar deze genen werden hier niet individueel onderzocht. Volgend onderzoek zou zich kunnen toespitsen op andere transportmechanismen voor de opname van ijzer, alsook op een complete deletie van de vermoedelijk ABC-transporter die betrokken is bij de opname van ijzer.

## REGULERINGVAN ARCHAELLA EXPRESSIE

Het vermogen om een plotselinge verandering in de omgeving van micro-organismen te detecteren en daarop te reageren behelst vaak regulerende systemen die gebruik maken van eiwit-fosforylering. In Archaea wordt het archaellum (voorheen: archaeaal flagellum), functioneel analoog aan het bacteriële flagellum, gebruikt wordt voor de beweeglijkheid van de cel en speelt daarmee een vitale rol in de meeste eencellige organismen.Het archaellum is gecodeerd in genomisch cluster van genen die gecontroleerd worden door twee promotors: *flab* en *flax* (Lassak *et al.*, 2012). In tegenstelling tot bij de Eurarchaeota wordt de regulering van archaellum-gestuurde beweeglijkheid slecht begrepen in crenarchaeale organismen als *Sulfolobus*-stammen. Noemenswaardig is daarbij dat de expressie en fabricage geïnduceerd wordt bij trypton-arme groeiomstandigheden in *S. acidocaldarius*.

Het *arn*-gencluster bevat de genen voor het Forkhead-associated FHA eiwit ArnA en het van Willebrand factor vWA eiwit ArnB, die altijd samen co-lokaliseren als genen. Deze twee eiwitten gaan waarschijnlijk een interactie met elkaar aan, maar beiden werden ook aangetroffen als onderdeel van de eerdergenoemde membraan-vesikels. Dit leidde tot vragen over hun mogelijke rol in een alternatief regulerings-netwerk, en hoe ze verbonden zijn met het ontstaan van deze vesikels. Om dit te onderzoeken werden de genen die coderen voor ArnA en ArnB verwijderd door middel van gen-deletie. Dit leidde tot een opmerkelijk resultaat; deze mutanten bleken een

defect te hebben in de productie van biofilm, waarbij een mogelijke connectie gemaakt kan worden met beweeglijkheid en/of het hechten aan oppervlakten. Voor een rol in membraan-vesikel productie was echter geen experimenteel bewijs. Gevoed door deze fundamentele observaties werd een vervolgstudie gestart naar de regulering van de expressie van het archaellum in *S. acidocaldarius*.

Beide ArnA en ArnB eiwitten worden gefosforyleerd *in vitro* door specifieke kinasen, welke ook meegezuiverd werden, wat duidt op een sterke interactie tussen deze eiwitten. Fosforylatie werd niet volledig uitgeschakeld door het muteren van een van de geconserveerde fosfopeptide-bindings FHA domein van ArnA, wat suggereert dat ArnA op meerdere posities gefosforyleerd kan worden. Helaas werden er geen andere posities gevonden waar ArnA en ArnB gefosforyleerd kunnen worden. Twee mogelijke verklaringen zijn daarbij niet uit te sluiten; of ArnB wordt gefosforyleerd door een endogeen systeem van *E. coli* dat gebruikt wordt als expressiesysteem, of niet-gefosforyleerd ArnB gaat een interactie aan met ArnA.

Een analyse van verschillende fenotypen lieten een negatief regulerend effect zien van ArnA en ArnB op de expressie van archaella. Gen-deleties van *arnA* en *arnB* lieten een verhoogde expressie zien van FlaB en FlaX, hyper-motiliteit op platen die opgewekt werd door een verhoogd aantal archaella. Deze observaties lijken samen te hangen met de regulering van de expressie van archaella, wat goed te rijmen is met de sterke interactie die deze eiwitten hebben. Deze resultaten vormen de eerste stap van het verkrijgen van meer inzicht in het netwerk van interacties dat ten grondslag ligt aan de regulering van de beweeglijkheid van Crenarchaeota en benadrukken de belangrijke rol die eiwit-fosforylatie heeft bij cellulaire processen in Archaea. De eventuele rol die ArnA en ArnB spelen in de productie van membraan-vesikels moet echter nog worden opgehelderd.

# 8

# 摘要与结论

## 引言

作为三大类微生物之一的古生菌，其分布广，生存在不同的生态环境中。对其研究兴趣主要表现在进化，适应和对外界应激反应机制，特别是涉及极端环境生长等方面。古生菌的形态学特征，各种新陈代谢和生活习性与细菌类似，而最基础的细胞功能包括复制，转录和翻译机制则与真核生物相似。同时古生菌在碳氮代谢方面也有其独有特性。硫化叶菌作为泉古菌的模式菌主要生长在高温(80 度)和酸性(pH 2-3)的火山温泉中。不同种类的硫化叶菌被分离同时序列被破译注释。目前大量新陈代谢的分析促进了对其功能的深入了解。这些发展促使了遗传手段的建立，使基因敲除和蛋白表达成为可能。特别是嗜酸热硫化叶菌，在黄石公园(Brock *et al.,* 1972)发现的一种嗜酸嗜热微生物，其在基因敲除体系的优越性使其成为遗传研究的最佳候选(Wagner *et al.,* 2009)并作为本论文的研究对象。

## 古生菌的细胞分裂

泉古菌的一个特性是其细胞表面能够分泌膜囊泡。这些囊泡被 S-层蛋白包裹，其膜脂质组成成分与细胞质膜相似。而一些蛋白大量存在于囊泡中，例如内吞体分选转运复合体（ESCRT-III）系统的同源蛋白和 Vps-4 蛋白片段，Vps-4 蛋白在真核生物中也是内吞体分选转运复合体系的一部分。尽管这些蛋白的确切起源未知，源自细胞表面的膜囊泡被认为与细胞分裂有关，因为在主要的泉古菌和几乎所有奇古菌中，内吞体分选转运复合体系统三同源蛋白对这一过程似乎是必要的。细胞分裂系统包括三个高保守基因，*cdvA*，*cdvB* 和 *cdvC* 三个基因在同一操纵子中，这一系统与真核生物的内吞体分选转运复合系统同源。CdvB 蛋白与 Vps24 同源，被推测是内吞体分选转运复合系统的核心组成。除了 *cdv* 操纵子外，大部分泉古菌存在多个 CdvB 同源蛋白，在嗜酸热硫化叶菌中被命名为 CdvB1-3，这三个 CdvB 同源蛋白在细胞循环，病毒感染中不同地表达，能够自体或相互间作用，但是与从 CdvB 蛋白不同，不能够和 CdvC 蛋白作用。由于 CdvB 蛋白的具体作用仍不清楚，因此本研究利用基因敲除手段探索其在细胞分裂中的功能作用。尽管敲除菌表现出生长缺陷，特别是 cdvB3 基因敲除菌出现严重的生长抑制并伴随着大细胞的形成和 DNA 不分裂的现象，然而除 *cdvB* 基因外，其他三个 *cdvB* 基因能够被敲除表明其并非起到不可替代作用。

没有 CdvB3 蛋白，已完成基因复制的细胞或因不能按时分裂而继续以缓慢速度生长，即使最终仍会分裂。这一表型与核心 *cdv* 基因低表达水平一致，基因的低表达可能引起多效性的生长迟缓抑制。尽管这些基因的低表达可能是低生长率，特别是固体培养基中菌生长缓慢的原因，但在 *cdvB3* 基因缺失菌液体培养基中，在对数生长中期，这些基因恢复正常表达水平，说明 CdvB3 蛋白并非起到主要作用，而更像是协同作用。

在野生型菌中，细胞分裂似乎与出现在细胞中部位置的 CdvA 蛋白组成的环状结构有关，该蛋白与膜绑定并且与 DNA 作用。这一膜绑定的蛋白环状结构进而促使 CdvB 蛋白形成相似的环状结构。随着环状结构收缩以及 CdvC 蛋白对 ATP 的水解利用，母细胞分裂成 2 个子细胞。细胞核分裂以及 CdvB 蛋白聚集的先决条件是 CdvA 蛋白的装配。就此而言，由于基因敲除菌缺失 CdvB3 蛋白，CdvA 蛋白似乎形成点状结构而非环状结构，很有可能这一结构无法促进 CdvB 蛋白的聚集组装。因此，在这些细胞里 CdvB 蛋白的荧光性很低，同时膜内陷现象也未在增大的细胞中发生。因此 CdvA 和 CdvB 蛋白异常的胞内位置进一步说明 CdvB3 蛋白在细胞分裂中协同作用。

另外两个 CdvB 同源蛋白的敲除并未引起明显的与细胞分裂相关的表型。有趣地，这些蛋白出现在嗜酸热硫化叶菌分泌的膜囊泡中，并且之前研究表明这些蛋白作用于病毒的分离。本研究数据表明 CdvB1 和 CdvB2 蛋白对细胞分裂不起主要作用，并且对囊泡形成的作用也尚不清楚。将来研究应放眼于细胞分裂与囊泡形成两者是否为关联过程，以及囊泡形成是否与病毒生源或者其他分泌形式是最初原始的现象。特别应引起兴趣的探索是研究缺陷菌 CdvB1 和 CdvB2 是否分泌和形成膜囊泡。就技术层面，这是一个苛刻的任务，因为即使在野生型菌中，在对数生长期囊泡的形成也很少，而对数生长晚期和稳定期，细胞裂解又会对分析产生影响。

## ABC-MDR 转运蛋白

对于泉古菌，ABC 转运蛋白是在营养贫瘠的极端环境中的一种重要生存策略。这种不利环境要求细胞采取进一步的保护机制，例如保护抵抗自然界中存在的或人为产生的有毒成分。ABC-MDR 转运蛋白在细胞保护方面特别有效，偶联 ATP 的水解给予细胞充分有效的抵抗浓度梯度。然而，几乎没有任何研究应用于古生菌的这一种转运蛋白。而事实情况是很多这类微生物生存在需要细胞保护抵抗不利环境中的有毒分子。目前为止，只有一个属于 SMR 家族的蛋白，Hsmr 蛋白被确认出现在嗜盐杆菌（Halobacterium salinarum）中，同时另一间接证据暗示一个 ABC-MDR 蛋白在沃氏嗜盐富饶菌（Haloferax volcanii）中具有抗药功能。生物信息分析表明在泉古菌嗜酸热硫化叶菌中同时存在一次和二次转运蛋白，几乎所有 ABC-MDR 转运蛋白是由一个 ABC 吸收系统和一个底物绑定蛋白组成，然而 *saci_2123* 基因（这里命名为 *smr1*），编码一个“半转运蛋白”，包含溶于 ATP 绑定区域 C 端的一个膜区域（章节 3），这一蛋白在古泉菌硫化叶菌中普遍存在。该蛋白表现出与其他已知细菌和哺乳动物 MDR 转运载体的高度同源性，这些蛋白不仅共享相似的核心结构，并且具备共同特性，例如具有识别和排出结构及功能不同的有毒底物的能力。

当细胞遭遇毒素时，*smr1* 基因表达水平上调，同时发现 *smr1* 基因敲除菌对一系列毒素比野生型菌耐受性差。当 Smr1 蛋白利用质粒系统超表达时,基因敲除菌的毒素敏感性可以被修复甚至高于野生型菌。这一现象表明已知抗药性是由 smr1 基因的表达而非相邻基因的极性效应决定。因此，Saci_2123 蛋白是第一个被初步确定具有抗药特性的 ABC 转运蛋白。未来研究兴趣应探索该蛋白在自然环境如高温酸性火山泉环境下的作用。然而由于 c 段 DUF1854 区域的存在，Smr1 蛋白明显地比细菌类的同源转运蛋白长，这一功能未知的区域可能作用于底物配基绑定。今后研究兴趣应检验去掉 C 端延伸的 Smr1 蛋白的细胞生长表型。另一方面，在布氏基础培养基中，基因敲除菌具有显著地表型变化，因此将来研究应致力于此基因敲除菌在硫化物被氧化成硫酸盐的无机化能营养生长条件下的表型。Smr1 蛋白也能够被用作遗传研究的筛选工具，遗传系统的发展在泉古菌目前依靠营养缺陷性标记，Smr1 蛋白筛选出的微毒素分子能够促进新的筛选标记的发展。

## ABC 铁绑定蛋白

铁是细胞内最丰富的金属元素，并且为多种代谢功能所需.环境中铁无法随意获得而是伴随其他分子或作为非溶解性盐，以氧化形式存在。目前大部分铁转运体统中，铁吸收依靠一个 ABC 转运蛋白和一个高专一底物亲和力的胞质绑定蛋白。对古生菌的铁转运吸收鲜有报道，目前只有嗜盐菌盐杆菌(Halobacterium)的含铁细胞的产生和铁吸收被详细报道 (Dave *et al.,* 2005; Hubmacher *et al.,* 2007)。嗜热嗜酸泉古菌的铁转运系统是研究热门，因为其酸性的生长环境为含铁细胞提供所需的易溶性的三价铁。事实上，硫化叶菌的含铁细胞无法在基础培养基上清液中获取，这与基因组未含有含铁细胞基因的观点一致。然而，两个不同含铁细胞种类的外源含铁细胞并不能促进菌生长或者铁吸收(章节 4)。三价铁的吸收符合典型的米门动力学，有趣地，生长于布氏基础培养基的细胞，其荧光标记铁的吸收是生长于无铁培养基的 2 倍多，说明与普遍发现的微生物生长在铁贫瘠环境下能够刺激高效铁吸收系统的表达不同。与之前对于其他嗜酸微生物的研究一致，在硫化叶菌基因组中只有很少数量的类铁转运蛋白被发现。生物信息分析揭示在三个类铁绑定蛋白中，其中一个与一个编码 ABC 转运蛋白基因在同一基因簇中。两个类铁绑定蛋白，其中与 ABC 转运蛋白相连的蛋白当细胞在铁贫瘠培养基生长时，其基因表达上调。绑定蛋白的敲除引起细胞在铁富集和铁贫瘠培养基中的生长抑制，同时影响铁吸收。在单基因敲除菌中，补偿性的基因水平表达似乎发生在抵消单敲除诱导的缺失，然而，双敲除和三敲除没有加深对菌的生长抑制，说明除了这些绑定蛋白机制，还有其他转运机制起主要作用，可能包括 NRAMP 家族的成员，但还未有实验证明。进一步研究应致力于对其他可能的转运机制以及对整个 ABC 转运蛋白基因簇的敲除。

## 鞭毛表达的调控

微生物察觉和对环境突变的反应能力包括可逆蛋白磷酸化的调控系统。古生菌的鞭毛功能类似于细菌鞭毛，应用于大部分单细胞生物最重要的功能：移动。编码鞭毛的基因簇由两个启动子 *flab* 和 *flax* (Lassak *et al.*, 2012) 调控。相对于广古菌，古泉菌生物例如硫化叶菌，对于鞭毛介导的移动调控机制仍旧不清楚。有趣地，嗜酸热硫化叶菌鞭毛表达和组装在胰蛋白胨限制生长条件下被诱导。arn 基因簇编码 Forkhead-associated FHA 蛋白 ArnA 以及 the von Willebrand factor vWA 蛋白 ArnB。这两个蛋白是互作蛋白，并都作为膜囊泡的成分出现。这引起的问题有：是否他们作用于可替代调节网络以及他们与囊泡形成的联系。因此，这两个基因作为敲除对象，显著地，基因敲除菌表现出生物膜形成缺陷可能与细胞移动和或者表面粘连依附有关。而对膜囊泡形成的功能无实验论证。这些基础的观察结果激起在鞭毛表达调节的进一步研究。

体外，两个蛋白被特定蛋白激酶磷酸化，并很强的互相作用。通过敲除 Arna 蛋白的一个保守的磷肽绑定 FHA 区域，磷酸化不能被完全屏蔽，说明 Arna 蛋白拥有不止一个磷酸化位点。不幸地，我们不能确定 ArnA 和 ArnB 蛋白的其他磷酸化位点。

表性分析揭示 ArnA 和 ArnB 蛋白在鞭毛表达的负调节作用。基因敲除菌展现了强鞭毛化诱导的 FlaB 和 FlaX 的高表达水平，平板培养基的运动过强，似乎两个蛋白调控鞭毛表达在同一等级水平，这与观察到的两者相互间的强作用一致。这些结果阐述了引起泉古菌细胞移动的调节网络的初步了解，详述了蛋白磷酸化在古生菌细胞过程调节的重要性。而 ArnA 和 ArnB 蛋白在膜囊泡形成中的作用仍需阐明。

&

APPENDIX

Supplementary material

Reference list

Acknowledgements

List of publications

# SUPPLEMENTARY MATERIAL

## TABLES

**Table S1 (all chapters).** Primers and probes used in this study.

| Primer | Sequence | Purpose |
|---|---|---|
| | **For chapter 2** | |
| 1 | CGCGCCGCGGTATTTTAAGGACTTGACGGAT | *ΔcdvB* US FW |
| 2 | GCCGGATCCGTATGTCTGCCCAAGTAATGCT | *ΔcdvB* US RV |
| 3 | GCCGGATCCCCTATGTCACTAATTCTTCATA | *ΔcdvB* DS FW |
| 4 | TCCCCCGGGTATTGCTCCATAAATTAACCC | *ΔcdvB* DS RV |
| 5 | TCCCCGCGGTGCTTAAGTTCTCCTGTCAGCAT | *ΔcdvB1* US FW |
| 6 | CGCGGATCCTTTTTCCCTCCCTTACCATTTTT | *ΔcdvB1* US RV |
| 7 | CGCGGATCCACCTCTTATAAAACAGAAGGTGC | *ΔcdvB1* DS FW |
| 8 | TCCCCCGGGCTCACTGAAACTTTCATCAATTT | *ΔcdvB1* DS RV |
| 9 | TCCCCGCGGATATTATACTCCAGCAGACTTTTAC | *ΔcdvB2* US FW |
| 10 | CGCGGATCCCTTCTATCTCGTAGTATATTCTTCC | *ΔcdvB2* US RV |
| 11 | CGCGGATCCATTCAATAATGAGATTTTTTG | *ΔcdvB2* DS FW |
| 12 | TCCCCCGGGTGTTCAACTTTAAGAGCTAGT | *ΔcdvB2* DS RV |
| 13 | TCCCCGCGGTCTGAGAGTTTACTATTAGTAT | *ΔcdvB3* US FW |
| 14 | CGCGGATCCAACTATATAGTGAAAATAGAGT | *ΔcdvB3* US RV |
| 15 | CGCGGATCCAAGTATCTTCTTTTAATCCC | *ΔcdvB3* DS FW |
| 16 | TCCCCCGGGTCCTTAGTGTAAACATGAAT | *ΔcdvB3* DS RV |
| 17 | CTCAATAGAGGACCAAGTTGC | qPCR *cdvA* fw |
| 18 | CTTCAGACTTATTACCCTCGTT | qPCR *cdvA* rv |
| 19 | ATAGCACCAGAGAAACTCCC | qPCR *cdvB* fw |
| 20 | TTGTCCAAAGCGATAACTGA | qPCR *cdvB* rv |
| 21 | CTTAACGTCTTCCAAACCCACGA | qPCR *cdvC* fw |
| 22 | AATGCTGAAGAGGCAATTACCAA | qPCR *cdvC* rv |
| 23 | TTACAGTCTCTAGCCTTAAA | qPCR *cdvB1* fw |
| 24 | AAGATTCCCAAGAGTAAA | qPCR *cdvB1* rv |
| 25 | AGAGAGATAGGAGCTTATTT | qPCR *cdvB2* fw |
| 26 | CCAACTCTATTGATAATTCA | qPCR *cdvB2* rv |
| 27 | GTAACTACATTCCCTAAA | qPCR *cdvB3* fw |
| 28 | GTGAAGAAAAGGACGA | qPCR *cdvB3* rv |
| 29 | CCCGATGTGATATAACTTACTAA | qPCR *secY* fw |
| 30 | GAGTCTTCTTTATTCGGATATGT | qPCR *secY* rv |
| | **For chapter 3** | |
| 31 | CGCGCCGCGGAAGCATTAGGTGATATAACA | *Δsmr1* US FW |
| 32 | GCCGGATCCATAAAGATTGGAAGATTTCT | *Δsmr1* US RV |
| 33 | GCCGGATCCACGATATTTGCAGATACTCTT | *Δsmr1* DS FW |
| 34 | TCCCCCGGGTGGATTCTCTACTGCTAATTT | *Δsmr1* DS RV |
| 35 | CATGCCATGGcgAAAAGAGAGTTAACAACAAC | *smr1* COM. FW |

**Table S1 (all chapters).** Primers and probes used in this study. (*Continued*)

| | | |
|---|---|---|
| 36 | CGCGGATCCTCAAATGGTCTCCAGTAATAATC | *smr1* COM. RV |
| 37 | TGAGGCAATGATGGAGGT | qPCR *smr1* fw |
| 38 | GCAGGAGTTATCTTAGTTGAC | qPCR *smr1* rv |
| | **For chapter 4** | |
| 39 | TCCCCGCGGGCTAAAACTGTCTGTTAACTTGA | *Δ saci 1220* US FW |
| 40 | CGCGGATCCGTGTAGTAGGATAGGCTATCCAT | *Δ saci 1220* US RV |
| 41 | CGCGGATCCATGAAAACCTTTAACAAAAT | *Δ saci 1220* DS FW |
| 42 | TCCCCCGGGGCAATTATGTTACTAAGCAA | *Δ saci 1220* DS RV |
| 43 | TCCCCGCGGTGGGGAGAGACTACACCGAGT | *Δ saci 2323* US FW |
| 44 | CGCGGATCCCCCTTTACGACCCCTAAGCATGT | *Δ saci 2323* US RV |
| 45 | CGCGGATCCTTTCTAATTCTCTTCTCTGGTT | *Δ saci 2323* DS FW |
| 46 | TCCCCCGGGCATTAGTGTCACAGAGCACTAT | *Δ saci 2323* DS RV |
| 47 | TCCCCGCGGTACTAGGATTAGAGAGCGTCTTC | *Δ saci 1488* US FW |
| 48 | CGCGGATCCTATTTTCTCTTTCTTAATTTGGC | *Δ saci 1488* US RV |
| 49 | CGCGGATCCATAGAGATATTAAAGCGTTTAG | *Δ saci 1488* DS FW |
| 50 | TCCCCCGGGAAAGACATAAGAGTAACTCAAG | *Δ saci 1488* DS RV |
| 51 | TGGATAGATAGTGATGGAACAG | qPCR *saci 1220* fw |
| 52 | CAACATCAGTAGCCAGACC | qPCR *saci 1220* rv |
| 53 | TTAACTTGGTAGGTGGTGAA | qPCR *saci 2323* fw |
| 54 | ACAAGGGATAACGCTATGC | qPCR *saci 2323* rv |
| 55 | CATCCTACAGCCTACATAATAG | qPCR *saci 1488* fw |
| 56 | CTACCTTAGAGCCGTTCAA | qPCR *saci 1488* rv |
| | **For chapter 5** | |
| | **primers for pSVA1031** | |
| 1575 | GGGCCATGGCTCTCCTGGAGGGATATTTC | *Δsaci1210* US FW |
| 1576 | ATAAACTCACTCCTTCCACGTCATACACGTAATATTCTG | *Δsaci1210* US RV |
| 1577 | ACGTGTATGACGTGGAAGGAGTGAGTTTATGACCATA | *Δsaci1210* DS FW |
| 1578 | GGGGGATCCATCTCGCCATACACTCTTAC | *Δsaci1210* DS RV |
| 1634 | TAGCTTACCTCGTTGATCAC | *Δsaci1210* check FW |
| 1635 | CGCTTTACATCATTTGCTCTTG | *Δsaci1210* check RV |
| | **primers for pSVA1062** | |
| 1579 | GGGCCATGGGCTTACCCTAACTATCAAAC | *Δsaci1211* US FW |
| 1580 | CCTTGTTTAAGACCTTATGGTCATAAACTCACTCCTT | *Δsaci1211* US RV |
| 1581 | GAGTTTATGACCATAAGGTCTTAAACAAGGACTAAATTTA TC | *Δsaci1211* DS FW |
| 1582 | GGGGATCCGTAGACATTGAAGAAGGTAAAG | *Δsaci1211* DS RV |
| 1636 | ATCTACTTGCTGAAGACAATG | *Δsaci1211* check FW |
| 1637 | GAACAGGGAGAGGACTTC | *Δsaci1211* check RV |
| | **primers for pSVA1069** | |
| 3118 | GGACCATGGCCTGAGGGTGAATCTGGCAAAC | *Δsaci1209* US FW |
| 1645 | GTTACCATAATACCTGAGTCAGTCTATCATAACAACGTAA TG | *Δsaci1209* US RV |

**Table S1 (all chapters).** Primers and probes used in this study. (*Continued*)

| | | |
|---|---|---|
| 1646 | TACGTTGTTATGATAGACTGACTCAGGTATTATGGTAACC TTATATC | *Δsaci1209* DS FW |
| 1647 | GGTGGATCCAGCCACTCGTCATTAATTAG | *Δsaci1209* DS RV |
| 1669 | ACAGACCCTACACCCTTCTC | *Δsaci1209* check FW |
| 1670 | GGTATCCTGCTCAATAGTTC | *Δsaci1209* check RV |
| | **primers for pSVA2217** | |
| 1575 | GGGCCATGGCTCTCCTGGAGGGATATTTC | *Δsaci1210* US FW |
| 3178 | TCCTTGTTTAAGACCTCCACGTCATACACGTAATATTCTG | *Δsaci1210 Δsaci1211* US RV |
| 3179 | ACGTGTATGACGTGGAGGTCTTAAACAAGGACTAAATTT ATC | *Δsaci1210 Δsaci1211* DS FW |
| 1582 | GGGGATCCGTAGACATTGAAGAAGGTAAAG | *Δsaci1211* DS RV |
| 1634 | TAGCTTACCTCGTTGATCAC | *Δsaci1210* check FW |
| 1637 | GAACAGGGAGAGGACTTC | *Δsaci1211* check RV |
| | **primers for pSVA1009** | |
| 1507 | GGGGTGATCAGGATTTAGCATTCGTTAAG | Saci1193 expr. FW |
| 1508 | GGGCTGCAGTTAGATCTCTGAGTAGTTAA | Saci1193 expr. RV |
| | **primers for pSVA1076** | |
| 1648 | GGGCCATGGAAAGTAGTAGTGTATC | Saci1694 expr. FW |
| 1649 | GGGCTGCAGTTAGTGGTGATGATGGTGATGTTTCTTCTCTG CTCTTTGTAATAAGTC | Saci1694 expr. RV |
| | **primers for pSVA1034** | |
| 1614 | GGGAGGGCATATGACGTGGAAATGTAATTTATGC | Saci1210 expr. FW |
| 1594 | GGGCCTAGGTTACTTCTCAAATTGTGGATGACTCCACTCC TTTAATATTCGTACTATTGTCTGATTACC | Saci1210 expr. RV Strep |
| | **primers for pSVA1036** | |
| 1597 | GGGCCATGGCTACCATATCAGTTAAAGCCGAATTAAG | Saci1211 expr. FW |
| 1598 | GGGGGATCCTTAGTGGTGATGATGGTGATGAGACCTCAA CTTCTTAGTAACTTCACTAG | Saci1211 expr. RV His |
| | **primers for pSVA1068** | |
| 1642 | GGACCATGGCCACAGTTAGCGTTTCTTTAAAATC | Saci1209 expr. FW |
| 1643 | GGCGGATCCTGTCCTCATTGTTTTTGTAACTTC | Saci1209 expr. RV |
| | **primers for pSVA2216** | |
| | Same as pSVA1034 and pSVA1036 in one plasmid | |
| | **primers for pSVA1037** | |
| 1599 | GGGCCATGGCTAACATTGAAGAAACGTATGAG | Saci0884 expr. FW |
| 1600 | GGGGGATCCTTAGTGGTGATGATGGTGATGTACTATCTCT TCTATTAGTTGATCGTTCAC | Saci0884 expr. RV His |
| | **primers for pSVA2234** | |
| 3133 | GTTTGCCATGGCGATAGTGAAGAAAGAG | Saci1193 $K_{393}$A FW |
| 3134 | CTTCACTATCGCCATGGCAAACTTTCTCATG | Saci1193 $K_{393}$A RV |
| | **primers for pSVA2238** | |
| 3141 | GTTAGCAGTAGCGATCTACAGGCTCGAG | Saci1694 $K_{137}$A FW |

**Table S1 (all chapters).** Primers and probes used in this study. (*Continued*)

| | | |
|---|---|---|
| 3142 | CCTGTAGATCGCTACTGCTAACTTATTAGC | Saci1193 $K_{137}$A RV |
| | **primers for pSVA2246** | |
| 3149 | ATCAATGGGTGCGAGTCCTGAAAAC | Saci1210 $R_{132}$A FW |
| 3150 | TTCAGGACTCGCACCCATTGATACAC | Saci1210 $R_{132}$A RV |
| | **primers for pSVA2247** | |
| 3151 | TTCAGAAGTAGCTAGAAAACACGCAG | Saci1210 $S_{146}$A FW |
| 3152 | GTGTTTTCTAGCTACTTCTGAATCAGG | Saci1210 $S_{146}$A RV |
| | **primers for pSVA1035** | |
| 1595 | GGGCCATGGCTACGTGGAAATGTAATTTATGC | Saci1210 hom. expr. FW |
| 1596 | GGGGGATCCCTCCTTTAATATTCGTACTATTG | Saci1210 hom. expr. RV |
| | **primers for pSVA2220** | |
| 1597 | GGGCCATGGCTACCATATCAGTTAAAGCCGAATTAAG | Saci1211 hom. expr. FW |
| 3187 | GGGGGATCCAGACCTCAACTTCTTAGTAACTTC | Saci1211 hom. expr. RV |
| | **primers for pSVA2254** | |
| 3236 | AGGCCGCGGATCTACTTGCTGAAGACAATG | *Δsaci1210_1211* COM. FW |
| 3237 | AGGCGGCCGTACGACGAGAAGGATAATACAG | *Δsaci1210_1211* COM. RV |

**Table S2.** Strains and plasmids used in this study.

| Strains | Genotype | Source/Reference |
|---|---|---|
| **Strains** | | |
| **DH5α** | *Escherichia coli* K-12 cloning strain l2 f80d/*lacZDM15* D(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (rK2 mK1) *supE44 thi-1 gyrA relA1* | Gibco |
| **BL21 (DE3)** | *Escherichia coli* expression strain | Stratagene |
| **RIL** | B F- *ompT* hsdS(rB- mB-) *dcm*+ Tetr *E. coli* gal λ (DE3) *endA* Hte [*argU ileY leuW* Camr] | |
| **Rosetta (DE3)** | *Escherichia coli* expression strain | Merck |
| **pLysS** | F- *ompT hsd*$S_B$($r_{B^-}$ $m_{B^-}$) *gal dcm* (DE3) pLysSRARE ($Cam^R$) | |
| **ER1821** | *Escherichia coli* propagation strain F- *glnV44 e14-(McrA-) rfbD1? relA1? endA1 spoT1? thi-1 Δ (mcrC-mrr)114::IS10* | New England Biolabs |
| **MW001** | *Sulfolobus acidocaldarius* DSM639 *ΔpyrE* | (Wagner et al., 2012) |
| **MW351** | MW001 *Δsaci1210* (*ΔarnA*) | This work |
| **MW353** | MW001 *Δ*saci*1211* (*ΔarnB*) | This work |
| **MW356** | MW001 *Δsaci1209* (*ΔvWA2*) | This work |
| **MW376** | MW001 *Δsaci1210Δsaci1211* (*ΔarnAΔarnB*) | This work |
| **MW156** | MW001 *Δsaci2318* (*ΔaapF*) | (Henche, Koerdt, Ghosh, & Albers, 2012) |
| **MW455** | MW001 *Δsaci2318Δsaci1174* (*ΔaapFΔflaH*) | (Lassak et al., 2012) |

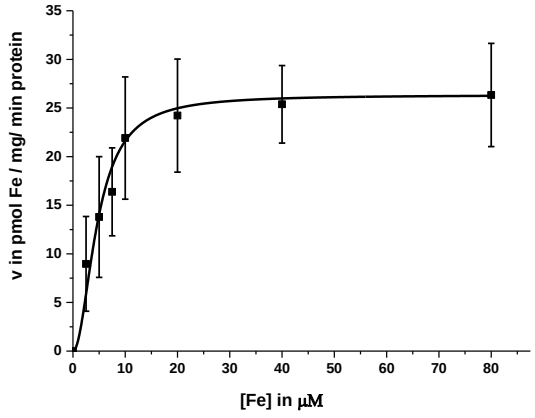
**Table S2.** Strains and plasmids used in this study. (*Continued*)

| | | |
|---|---|---|
| **Plasmids** | | |
| **pSVA406** | Gene targeting plasmid, pGEM-T Easy backbone, $pyrEF_{SSO}$ cassette; single crossover method | (Wagner et al., 2012) |
| **pSVA407** | Gene targeting plasmid, pGEM-T Easy backbone, $pyrEF_{SSO}$ and $lacS_{SSO}$ cassette; single crossover method | (Wagner et al., 2012) |
| **pΔ2*pyrEF*** | Gene targeting plasmid, pBluescript backbone, $pyrEF_{SSO}$; single crossover method | (Wagner et al., 2009) |
| **pSVA1031** | In-frame deletion of *saci1210* (*arnA*) cloned into pSVA406 with *Nco*I, *Bam*HI | This work |
| **pSVA1062** | In-frame deletion of *saci1211* (*arnB*) cloned into pSVA407 with *Nco*I, *Bam*HI | This work |
| **pSVA1069** | In-frame deletion of *saci1209* (*vWA2*) cloned into pSVA406 with *Nco*I, *Bam*HI | This work |
| **pSVA2217** | In-frame deletion of *saci1210* (*arnA*) and *saci1211* (*arnB*) cloned into pSVA406 with *Nco*I, *Bam*HI | This work |
| **pETDuet-1** | $Amp^r$, $Car^r$, expression plasmid containing replicon ColE1 (pBR322) and two MCS (MCS1 and MCS2) | Novagen |
| **pSA4** | Derivative of pET15b containing the multiple cloning site and C-terminal hexa-His tag of pSA5 | (Albers, Szabó, & Driessen, 2003) |
| **pSVA1009** | *saci1193* with N-terminal His-tag cloned into pETDuet-1 with *Bcl*I/*Bam*HI, *Pst*I into MCSI | This work |
| **pSVA1076** | *saci1694* with C-termial His-tag cloned into pETDuet-1 with *Nco*I, *Pst*I in MCSI | This work |
| **pSVA1034** | *saci1210* with C-terminal Strep-tag cloned into pETDuet-1 with *Nde*I, *Avr*II in MCSII | This work |
| **pSVA1036** | *saci1211* with C-termial His-tag cloned into pETDuet-1 with *Nco*I, *Bam*HI in MCSI | This work |
| **pSVA1068** | *saci1209* with C-termial His-tag cloned into pSA4 with *Nco*I, *Bam*HI | This work |
| **pSVA2216** | *saci1211* with C-termial His-tag cloned into pETDuet-1 with *Nco*I, *Bam*HI in MCSI and *saci1210* with C-terminal Strep-tag cloned into pETDuet-1 with *Nde*I, *Avr*II in MCSII | This work |
| **pSVA1037** | *Saci0884* with C-termial His-tag cloned into pETDuet-1 with *Nco*I, BamHI in MCSI | This work |
| **pSVA2234** | *saci1193* K393A with N-terminal His-tag in pETDuet-1 mutated from pSVA1009 | This work |
| **pSVA2238** | *saci1694* K137A with C-termial His-tag in pETDuet-1 mutated from pSVA1076 | This work |
| **pSVA2246** | *saci1210* R132A with C-terminal Strep-tag in pETDuet-1 mutated from pSVA1034 | This work |
| **pSVA2247** | *saci1210* S146A with C-terminal Strep-tag in pETDuet-1 mutated from pSVA1034 | This work |
| **pSVA2254** | *saci1210_1211* with own promoter and stop region for | This work |

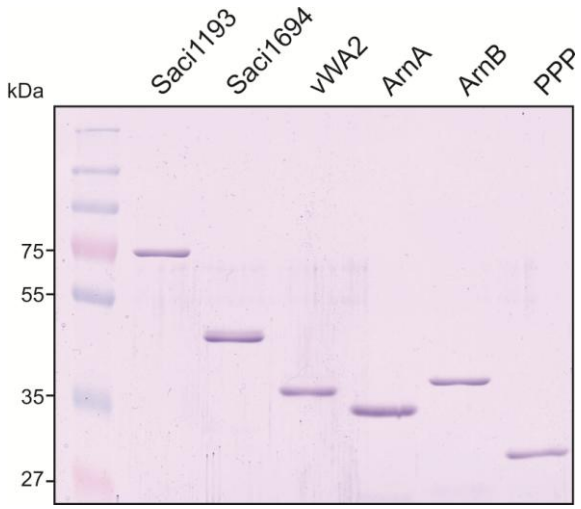
**Table S2.** Strains and plasmids used in this study. (*Continued*)

| | | |
|---|---|---|
| | complementation of Δ*saci1210*Δ*1211* (MW376) | |
| **pMZ1** | *E. coli* entry vector with *ara*-promoter and C-terminal Strep- and His-tag | (Zolghadr, Weber, Szabo, Driessen, & Albers, 2007) |
| **pCMalLacS** | pRN-1 based shuttle vector with $lacS_{SSO}$ reporter gene | (Berkner, Wlodkowski, Albers, & Lipps, 2010) |
| **pSVA1481** | *E. coli* entry vector with *ara*-promoter and C-terminal Strep- and His-tag based on pGEM-T Easy backbone and pMZ1 cassette | Wagner & Albers, unpublished |
| **pSVA1450** | Plasmid for expression in *S. acidocaldarius* based on pCMalLacS with *mal* promoter | Wagner & Albers, unpublished |
| **pSVA1035** | *saci1210* cloned into pMZ1 with *Nco*I, *Bam*HI | This work |
| **pSVA2220** | *saci1211* cloned into pSVA1481 with *Nco*I, *Bam*HI | This work |
| **pSVA1066** | *saci1210* cloned from pSVA1035 into pCMalLacS with *Nco*I, *Eag*I | This work |
| **pSVA2222** | *saci1210* cloned from pSVA1035 into pSVA1450 with *Nco*I, *Eag*I | This work |
| **pSVA2221** | *saci1211* cloned from pSVA2220 into pSVA1450 with *Nco*I, *Eag*I | This work |
| **pSVA1600** | *flaB* promoter replacing *mal* promoter, cloned into pCMalLacS with *Sac*II, *Nco*I | (Lassak et al., 2012) |
| **pSVA1601** | *flaX* promoter replacing *mal* promoter, cloned into pCMalLacS with *Sac*II, *Nco*I | (Lassak et al., 2012) |
| **pSVA1614** | pClacS, promoterless vector for negative control in promoter activity assay | (Lassak et al., 2012) |

# FIGURES



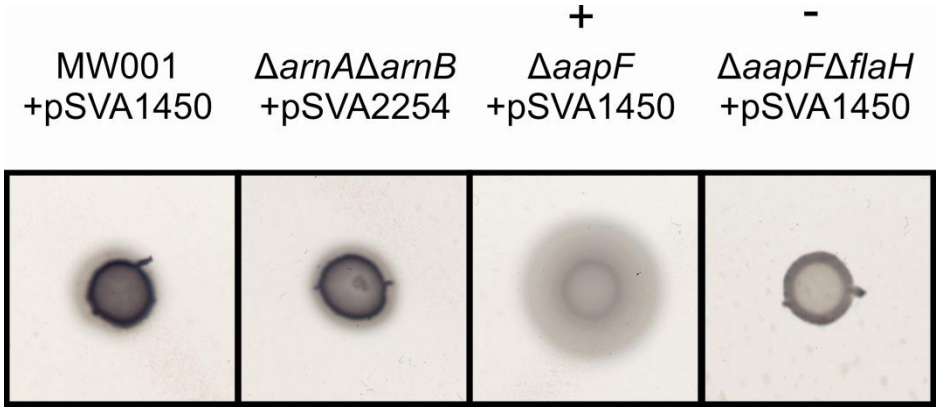

**Chapter 4 Figure S1.** Concentration dependent uptake of iron into *Sulfolobus* cells. Iron-uptake shows saturable kinetics. The mean of n = 3 experiments with double determinations is shown including standard deviations.



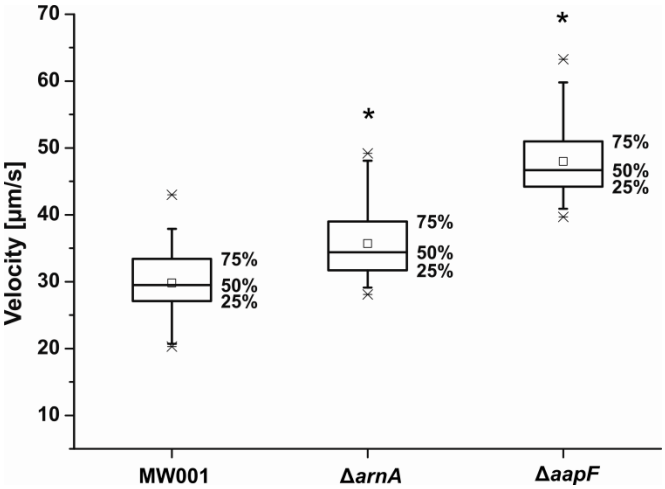

**Chapter 5 Figure S1.** Expression and purification of the kinases Saci1193 and Saci1694, ArnA, ArnB vWA2, and the phosphatase PPP. All proteins were expressed in *E. coli* BL21 (DE3) RIL or Rosetta (DE3) pLysS with an IPTG inducible T7 promoter. The proteins were purified either via Ni-NTA column or via Strep-Avidin column depending on the tag. Increased purity was obtained with gel filtration.

```
                                          132       146
Sulfolobus acidocaldarius  ArnA         LFPSVSMGRSPENIVIVPDSEVSRKHAVIYLD----NSELYIEDLNSTNGTYVYDGKQFTPIKGKQKIEPNSIIKLGNQTIVRILKE-- 206
Sulfolobus solfataricus    SSO1088      IFPSISIGRSPENVIVIPDPEVSRKHAIISFE----NNELYLEDLNSTNGTYVYDGKVFQPVKGKIKVQHNSIIKLGNNTVVRIVGE-- 209
Sulfolobus tokodaii        ST0829       IFPSISVGRSPENVIIVPDPEVSRRHALISLE----GGELYIEDLNSTNGTYIYDGKLFQPVKGKQKISPNTIIKLGNQTIVKVLAE-- 238
Metallosphaera sedula      Msed_1490    VFENISLGRSPENVLMIPDSEISRRHAILSKE----GDTLYIEDLNSTNGTYIYDGKVFQTVKGKVQLPKNAVVKLGNNTIIKIVRE-- 218
Picrophilus torridus       PTO0287      VFPEITIGRSPENVIVVPDADVSKKHAVIRMN----NNVIEIEDLNSTNGTFLYDGK-FVKLTGKTPLKNNALIRFGYSTMFKLVFD-- 255
Acidianus hospitalis       Ahos_0924    VFESISIGRSPENVIVIPDPEISRRHAIISLE----GGKLYIEDLNSTNGTYLYDGKLFQPVKGKQELAPGSIIKLGSNTMLKIVIE-- 233
Mycobacterium tuberculosis Rv0020c      REGSNIIGRGQDAQFRLPDTGVSRRHLEIRWD----GQVALLADLNSTNGTTVNNAP-----VQEWQLADGDVIRLGHSEIIVRMH--- 527
Mycobacterium tuberculosis Rv1827       ---ITSAGRHPDSDIFLDDVTVSRRHAEFRLE----NNEFNVVDVGSLNGTYVNREP-----VDSAVLANGDEVQIGKFRLVFLTGPKQ 162
Saccharomyces cerevisiae   Rad53 FHA2  -VNPFFIGRSEDCNCKIEDNRLSRVHCFIFKKRHAVGKSMYESPAQGLDDIWYCHTGTNVSYLNNNRMIQGTKFLLQDGDEIKIIWDKN 685
                                          **  :    : *  :*: *  :  .    .        . :.           .  :  .  . :    .
```

**Chapter 5 Figure S2.** Alignment of FHA-domains from different organisms. The most conserved amino acid residues of the FHA domains are colored in gray. The numbers above indicate the amino acid position in ArnA that were mutated to an alanine residue.




**Chapter 5 Figure S3.** Motility assay of the trans-complemented hypermotile strain Δ*arnA*Δ*arnB*. Strains were grown for five days on semi-solid gelrite plates with 0.005 % NZ-Amine and 0.2% D-maltose, but without Uracil. Δ*arnA*Δ*arnB* was transformed with pSVA2254 containing the *arnAarnB*-operon with its own promoter and terminator region. The control strains (*ΔaapF* and *ΔaapF*Δ*flaH*) and the background strain MW001 were transformed with the expression vector pSVA1450 to be able to grow without uracil. The hypermotile phenotype of Δ*arnA*Δ*arnB* was complemented and showed motility comparable to MW001.




**Chapter 5 Figure S4.** Swimming velocities of MW001, *ΔarnA* and *ΔaapF*. Swimming velocities were determined by tracking and evaluating single cell movement from thermomicroscopy movies via ImageJ. The diagram shows a box plot, in which the middle 50 % of the values are depicted in the box. The line shows the median and the square indicates the mean value, while the star (*) shows the significance in comparison to the wild type strain. The *arnA* deletion strain has an intermediate velocity (35 µm $s^{-1}$) compared to the background strain MW001 and the hypermotile strain *ΔaapF*.

# REFERENCE LIST


Alatyrev, A. G., Pyatibratov, M. G., Kawarabayasi, Y., Tsujimura, M., Galeva, A. V. and Fedorov, O. V. (2010). Identification of the new protein participating in the archaea motility regulation. Biochem Mosc Suppl Ser A Membr Cell Biol 4, 104-114.

Albers, S. V., Elferink, M. G., Charlebois, R. L., Sensen, C. W., Driessen, A. J. and Konings, W. N. (1999). Glucose transport in the extremely thermoacidophilic *Sulfolobus solfataricus* involves a high-affinity membrane-integrated binding protein. J Bacteriol 181, 4285-4291.

Albers S. V., Koning S. M., Konings W. N. and Driessen A. J. (2004). Insights into ABC transport in archaea. J Bioenerg Biomembr 36, 5-15.

Albers, S. V. and Meyer, B. H. (2011). The archaeal cell envelope. Nat Rev Microbiol 9, 414-426.

Allers, T. and Mevarech, M. (2005). Archaeal genetics-the third way. Nat Rev Genet 6, 58-73.

Andersson, A., Bernander, R. and Nilsson, P. (2005). Dual-genome primer design for construction of DNA microarrays. Bioinformatics 21, 325-332.

Andrews, S. C., Robinson, A. K. and Rodriguez-Quinones, F. (2003). Bacterial iron homeostasis. FEMS Microbiol Rev 27, 215-237.

Angerer, A., Gaisser, S. and Braun, V. (1990). Nucleotide sequences of the *sfuA*, *sfuB*, and *sfuC* genes of *Serratia marcescens* suggest a periplasmic-binding-protein-dependent iron transport mechanism. J Bacteriol 172, 572-578.

Babst, M. (2005). A protein's final ESCRT. Traffic 6, 2-9.

Babst M., Wendland B., Estepa E. J. and Emr S. D. (1998). The Vps4p AAA ATPase regulates membrane association of a vps protein complex required for normal endosome function. EMBO J 17, 2982-2993.

Baker, B. J., Tyson, G. W., Webb, R. I., Flanagan, J., Hugenholtz, P., Allen, E. E. and Banfield, J. F. (2006). Lineages of acidophilic archaea revealed by community genomic analysis. Science 314, 1933-1935.

Baker, B. J., Comolli, L. R., Dick, G. J. and other authors (2010). Enigmatic, ultrasmall, uncultivated Archaea. Proc Natl Acad Sci U. S. A. 107, 8806-8811.

Bandyopadhyay, S., Chandramouli, K. and Johnson, M. K. (2008). Iron-sulfur cluster biosynthesis. Biochem Soc Trans 36, 1112-1119.

Barns, S. M., Fundyga, R. E., Jeffries, M. W. and Pace, N. R. (1994). Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc Natl Acad Sci U. S. A. 91, 1609-1613.

Barns, S. M., Delwiche, C. F., Palmer, J. D. and Pace, N. R. (1996). Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. Proc Natl Acad Sci U. S. A. 93, 9188-9193.

Bartolucci, S., Rossi, M. and Cannio, R. (2003). Characterization and functional complementation of a nonlethal deletion in the chromosome of a beta-glycosidase mutant of *Sulfolobus solfataricus*. J Bacteriol 185, 3948-3957.

Bell, S. D. and Jackson, S. P. (2001). Mechanism and regulation of transcription in archaea. Curr Opin Microbiol 4, 208-213.

Berkner, S., Grogan, D., Albers, S. V. and Lipps, G. (2007). Small multicopy, non-integrative shuttle vectors based on the plasmid pRN1 for *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus*, model organisms of the (cren-)archaea. Nucleic Acids Res 35, e88.

Berkner, S. and Lipps, G. (2008). Genetic tools for *Sulfolobus* spp.: vectors and first applications. Arch Microbiol 190, 217-230.

Berkner, S., Wlodkowski, A., Albers, S. V. and Lipps, G. (2010). Inducible and constitutive promoters for geneticsystems in *Sulfolobus acidocaldarius*. Extremophiles 14, 249–259.

Bernander, R. (2000). Chromosome replication, nucleoid segregation and cell division in archaea. Trends Microbiol 8, 278-283.

Bini, E. (2000). Archaeal transformation of metals in the environment. FEMS Microbiol Ecol 73, 1-16.

Bintrim, S. B., Donohue, T. J., Handelsman, J., Roberts, G. P. and Goodman, R. M. (1997). Molecular phylogeny of Archaea from soil. Proc Natl Acad Sci U. S. A. 94, 277-282.

Blochl, E., Rachel, R., Burggraf, S., Hafenbradl, D., Jannasch, H. W. and Stetter, K. O. (1997). *Pyrolobus fumarii*, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113 degrees C. Extremophiles 1, 14-21.

Boos, W. (1974). Bacterial transport. Annu Rev Biochem 43, 123-146.

Borths, E. L., Locher, K. P., Lee, A. T. and Rees, D. C. (2002). The structure of *Escherichia coli* BtuF and binding to its cognate ATP binding cassette transporter. Proc Natl Acad Sci U. S. A. 99, 16642-16647.

Brochier, C., Gribaldo, S., Zivanovic, Y., Confalonieri, F. and Forterre, P. (2005). Nanoarchaea: representatives of a novel archaeal phylum or a fast-evolving euryarchaeal lineage related to Thermococcales? Genome Biol 6, R42.

Brochier-Armanet, C., Boussau, B., Gribaldo, S. and Forterre, P. (2008). Mesophilic Crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeota. Nat Rev Microbiol 6, 245-252.

Brock, T. D., Brock, K. M., Belly, R. T. and Weiss, R. L. (1972). *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. Arch Microbiol 84, 54-68.

Buckley, D. H., Graber, J. R. and Schmidt, T. M. (1998). Phylogenetic analysis of nonthermophilic members of the kingdom crenarchaeota and their diversity and abundance in soils. Appl Environ Microbiol 64, 4333-4339.

Bush, K. and Miller, G. H. (1998). Bacterial enzymatic resistance: Beta-lactamases and aminoglycoside-modifying enzymes. Curr Opin Microbiol 1, 509-515.

Cameron, V., House, C. H. and Brantley, S. L. (2012). A first analysis of metallome biosignatures of hyperthermophilic Archaea. Archaea 2012, 789278.

Carlton, J. (2010). The ESCRT machinery: A cellular apparatus for sorting and scission. Biochem Soc Trans 38, 1397-1412.

Carlton, J. G. and Martin-Serrano, J. (2007). Parallels between cytokinesis and retroviral budding: A role for the ESCRT machinery. Science 316, 1908-1912.

Carlton, J. G. and Martin-Serrano, J. (2009). The ESCRT machinery: new functions in viral and cellular biology. Biochem Soc Trans 37, 195-199.

Cserháti, M., Kriszt, B., Szoboszlay, S., Tóth, Á., Szabó, I., Táncsics, A., Nagy, I., Horváth, B., Nagy, I. and Kukolya, J. (2012). De novo genome project of *Cupriavidus basilensis* OR16. J Bacteriol 194, 2109-2110.

Chen, J., Sharma, S., Quiocho, F. A. and Davidson, A. L. (2001). Trapping the transition state of an ATP-binding cassette transporter: evidence for a concerted mechanism of maltose transport. Proc Natl Acad Sci U. S. A. 98, 1525-1530.

Chen, J., Lu, G., Lin, J., Davidson, A. L. and Quiocho, F. A. (2003). A tweezers-like motion of the

ATP-binding cassette dimer in an ABC transport cycle. Mol Cell 12, 651-661.

Church, M. J., DeLong, E. F., Ducklow, H. W., Karner, M. B., Preston, C. M. and Karl, D. M. (2003). Abundance and distribution of planktonic Archaea and Bacteria in the waters west of the Antarctic Peninsula. Limnol Oceanogr 48, 1893-1902.207.

Clarke, T. E., Braun, V., Winkelmann, G., Tari, L. W. and Vogel, H. J. (2002). X-ray crystallographic structures of the *Escherichia coli* periplasmic protein FhuD bound to hydroxamate-type siderophores and the antibiotic albomycin. J Biol Chem 277, 13966-13972.

Colombatti, A., Bonaldo, P. and Doliana, R. (1993). Type Amodules: interacting domains found in several non-fibrillarcollagens and in other extracellular matrix proteins. Matrix 13, 297-306.

Conrad, M. E., Umbreit, J. N., Moore, E. G., Hainsworth, L. N., Porubcin, M., Simovich, M. J., Nakada, M. T., Dolan, K. and Garrick, M. D. (2000). Separate pathways for cellular uptake of ferric and ferrous iron. Am J Physiol Gastrointest Liver Physiol 279, G767-774.

Cyert, M. S. and Philpott, C. C. (2013). Regulation of cation balance in *Saccharomyces cerevisiae*. Genetics 193, 677-713.

Dassa, E. and Hofnung, M. (1985). Sequence of gene malG in E. coli K12: homologies between integral membrane components from binding protein-dependent transport systems. EMBO J 4, 2287-2293.

Dave, B. P., Anshuman, K. and Hajela, P. (2006). Siderophores of halophilicarchaea and their chemical characterization. Indian J Exp Biol 44, 340-344.

Davidson, A. L. (2002). Mechanism of coupling of transport to hydrolysis in bacterial ATP-binding cassette transporters. J Bacteriol 184, 1225-1233.

Dean, D. A., Davidson, A. L. and Nikaido, H. (1989). Maltose transport in membrane vesicles of *Escherichia coli* is linked to ATP hydrolysis. Proc Natl Acad Sci U. S. A. 86, 9134-9138.

Deatherage, B. L., Lara, J. C., Bergsbaken, T., Rassoulian Barrett, S. L., Lara, S. and Cookson, B. T. (2009). Biogenesis of bacterial membrane vesicles. Mol Microbiol 72, 1395-1407.

Deng, L., Zhu, H., Chen, Z., Liang, Y. X. and She, Q. (2009). Unmarked gene deletion and host-vector system for the hyperthermophilic crenarchaeon *Sulfolobus islandicus*. Extremophiles 13, 735-746.

Diederichs, K., Diez, J., Greller, G., Muller, C., Breed, J., Schnell, C., Vonrhein, C., Boos, W. and Welte, W. (2000). Crystal structure of MalK, the ATPase subunit of the trehalose/maltose ABC transporter of the archaeon *Thermococcus litoralis*. EMBO J 19, 5951-5961.

Diez, J., Diederichs, K., Greller, G., Horlacher, R., Boos, W. and Welte, W. (2001). The crystal structure of a liganded trehalose/maltose-binding protein from the hyperthermophilic Archaeon *Thermococcus litoralis* at 1.85 A. J Mol Biol 305, 905-915.

Dobro, M. J., Samson, R. Y., Yu, Z., McCullough, J., Ding, H. J., Chong, P. L., Bell, S. D. and Jensen, G. J. (2013). Electron cryotomography of ESCRT assemblies and dividing *Sulfolobus* cells suggests that spiraling filaments are involved in membrane scission. Mol Biol Cell 24, 2319-2327.

Donald, A., McHardy, T., Rowlands, M. G. and other authors (2007). Rapid evolution of 6-phenylpurine inhibitors of protein kinase B through structure-based design. J Biol Chem 50, 2289-2292.

Drazek, E. S., Hammack, C. A. and Schmitt, M. P. (2000). Corynebacterium diphtheriae genes required for acquisition of iron from haemin and haemoglobin are homologous to ABC haemin transporters. Mol Microbiol 36, 68-84.

Dridi, B., Fardeau, M. L., Ollivier, B., Raoult, D. and Drancourt, M. (2011). The antimicrobial resistance pattern of cultured human methanogens reflects the unique phylogenetic position of archaea. J Antimicrob Chemother 66, 2038–2044.

Duan, X. and He, Z. G. (2011). Characterization of the specificinteraction between archaeal FHA domain-containingprotein and the promoter of a flagellar-like gene-cluster and its regulation by phosphorylation. Biochem Biophys Res Commun 407, 242–247.

Duggin, I. G., McCallum, S. A. and Bell, S. D. (2008). Chromosome replication dynamics in the archaeon *Sulfolobus acidocaldarius*. Proc Natl Acad Sci U. S. A. 105, 16737-16742.

Durocher, D., Taylor, I. A., Sarbassova, D., Haire, L. F., Westcott,S. L., Jackson, S. P. and other authors (2000). The molecular basis of FHA domain: phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms. Mol Cell 6, 1169-1182.

Dwyer, M. A. and Hellinga, H. W. (2004). Periplasmic binding proteins: a versatile superfamily for protein engineering. Curr Opin Struct Biol 14, 495-504.

Dyer, C. M., Vartanian, A. S., Zhou, H. and Dahlquist, F. W. (2009). A molecular mechanism of bacterial flagellar motor switching. J Mol Biol 388, 71-84.

Eichler, J. (2003). Facing extremes: archaeal surface-layer (glyco) proteins. Microbiology 149, 3347-3351.

Elferink, M. G., de Wit, J. G., Driessen, A. J. and Konings, W. N. (1994). Stability and proton-permeability of liposomes composed of archaeal tetraether lipids. Biochim Biophys Acta 1193, 247-254.

Elferink, M. G., Albers, S. V., Konings, W. N. and Driessen, A. J. (2001). Sugar transport in *Sulfolobus solfataricus* is mediated by two families of binding protein-dependent ABC transporters. Mol Microbiol 39, 1494-1503.

Elkins, J. G., Podar, M., Graham, D. E. and other authors (2008). A korarchaeal genome reveals insights into the evolution of the Archaea. Proc Natl Acad Sci U. S. A. 105, 8102-8107.

Ellen, A. F., Albers, S. V., Huibers, W., Pitcher, A., Hobel, C. F., Schwarz, H., Folea, M., Schouten, S., Boekema, E. J., Poolman, B. and Driessen, A. J. (2009). Proteomic analysis of secreted membrane vesicles of archaeal *sulfolobus* species reveals the presence of endosome sorting complex components. Extremophiles 13, 67-79.

Ellen, A. F., Albers, S. V. and Driessen, A. J. (2010). Comparative study of the extracellular proteome of *Sulfolobus* species reveals limited secretion. Extremophiles 14, 87-98.

Ellen, A. F., Rohulya, O. V., Fusetti, F., Wagner, M., Albers, S.V. and Driessen, A. J. (2011). The sulfolobicin genes of *sulfolobus acidocaldarius* encode novel antimicrobial proteins. J Bacteriol 193, 4380-4387.

Ellen, A. F., Zolghadr, B., Driessen, A. M. and Albers, S. V. (2010). Shaping the archaeal cell envelope. Archaea, 608243.

Engelhardt, H. (2007). Are S-layers exoskeletons? The basic function of protein surface layers revisited. J Struct Biol 160, 115-124.

Erra-Pujada, M., Debeire, P., Duchiron, F. and O'Donohue, M. J. (1999). The type II pullulanase of *Thermococcus hydrothermalis*: molecular characterization of the gene and expression of the catalytic domain. J Bacteriol 181, 3284-3287.

Ettema, T. J. and Bernander, R. (2009). Cell division and the ESCRT complex: A surprise from the archaea. Commun Integr Biol 2, 86-88.

Evdokimov, A. G., Anderson, D. E., Routzahn, K. M. and Waugh, D. S. (2001). Structural basis for

oligosaccharide recognition by *Pyrococcus furiosus* maltodextrin-binding protein. J Mol Biol 305, 891-904.

Expert, D., Enard, C. and Masclaux, C. (1996). The role of iron in plant host-pathogen interactions. Trends Microbiol 4, 232-237.

Fiala, G. and Stetter, K. O. (1986). *Pyrococcus furiosus* sp. nov. represents a novel genius of marine heterotrophic archaebacteria growing optimally at 100°C. Arch.Microbiol 145, 56-61.

Futterer, O., Angelov, A., Liesegang, H., Gottschalk, G., Schleper, C., Schepers, B., Dock, C., Antranikian, G. and Liebl, W. (2004). Genome sequence of *Picrophilus torridus* and its implications for life around pH 0. Proc Natl Acad Sci U. S. A. 101, 9091-9096.

Genco, C. A. and Desai, P. J. (1996). Iron acquisition in the pathogenic *Neisseria*. Trends Microbiol 4, 179-184.

Genco, C. A. and Dixon, D. W. (2001). Emerging strategies in microbial haem capture. Mol Microbiol 39, 1-11.

Ghazi-Tabatabai, S., Obita, T., Pobbati, A. V., Perisic, O., Samson, R. Y., Bell, S. D. and Williams, R. L. (2009). Evolution and assembly of ESCRTs. Biochem Soc Trans 37, 151-155.

Gindner, A., Hausner, W. and Thomm, M. (2014). The TrmB family: a versatile group of transcriptional regulators in Archaea. Extremophiles 18, 925-936.

Gotz, D., Paytubi, S., Munro, S., Lundgren, M., Bernander, R. and White, M. F. (2007). Responses of hyperthermophilic crenarchaea to UV irradiation. Genome Biol 8, R220.

Grabowski, B. and Kelman, Z. (2003). Archeal DNA replication: eukaryal proteins in a bacterial context. Annu Rev Microbiol 57, 487-516.

Greller, G., Riek, R. and Boos, W. (2001). Purification and characterization of the heterologously expressed trehalose/maltose ABC transporter complex of the hyperthermophilic archaeon *Thermococcus litoralis*. Eur J Biochem 268, 4011-4018.

Grimm, R., Singh, H., Rachel, R., Typke, D., Zillig, W. and Baumeister, W. (1998). Electron tomography of ice-embedded prokaryotic cells. Biophys J 74, 1031-1042.

Grundner, C., Gay, L. M. and Alber, T. (2005). *Mycobacterium tuberculosis* serine/threonine kinases PknB, PknD, PknE, and PknF phosphorylate multiple FHA domains. Protein Sci 14, 1918-1921.

Guo, L., Brugger, K., Liu, C. and other authors (2011). Genome analyses of Icelandic strains of *Sulfolobus islandicus*, model organisms for genetic and virus-host interaction studies. J Bacteriol 193, 1672-1680.

Guy, L. and Ettema, T. J. (2011). The archaeal 'TACK' superphylum and the origin of eukaryotes. Trends Microbiol 19, 580-587.

Henche, A. L., Koerdt, A., Ghosh, A. and Albers, S. V. (2012). Influence of cell surface structures on crenarchaeal biofilm formation using a thermostable green fluorescent protein. Environ Microbiol 14, 779-793.

Hendrickson, E. L., Liu, Y., Rosas-Sandoval, G., Porat, I., Söll, D., Whitman, W. B. and Leigh, J. A. (2008). Global responses of *Methanococcus maripaludis* to specific nutrient limitations and growth rate. J Bacteriol 190, 2198-2205.

Henne, W. M., Buchkovich, N. J. and Emr, S. D. (2011). The ESCRT pathway. Dev Cell 21, 77-91.

Hentze, M. W., Muckenthaler, M. U., Galy, B. and Camaschella, C. (2010). Two to tango: regulation of Mammalian iron metabolism. Cell 142, 24-38.

Hershberger, K. L., Barns, S. M., Reysenbach, A. L., Dawson, S. C. and Pace, N. R. (1996). Wide

diversity of Crenarchaeota. Nature 384, 420.

Hettmann, T., Schmidt, C. L., Anemuller, S., Zahringer, U., Moll, H., Petersen, A. and Schafer, G. (1998). Cytochrome b558/566 from the archaeon *Sulfolobus acidocaldarius*. A novel highly glycosylated, membrane-bound b-type hemoprotein. J Biol Chem 273, 12032-12040.

Higgins, C. F. (1992). ABC transporters: from microorganisms to man. Annu Rev Cell Biol 8, 67-113.

Higgins, C. F. (2001). ABC transporters: physiology, structure and mechanism--an overview. Res Microbiol 152, 205-210.

Higgins, C. F. (2007). Multiple molecular mechanisms for multidrug resistance transporters. Nature 446, 749-57.

Higgins, C. F., Hiles, I. D., Salmond, G. P. and other authors (1986). A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. Nature 323, 448-450.

Hjort, K. and Bernander, R. (1999). Changes in cell size and DNA content in *Sulfolobus* cultures during dilution and temperature shift experiments. J Bacteriol 181, 5669-5675.

Hobel, C. F., Albers, S. V., Driessen, A. J. and Lupas, A. N. (2008). The *sulfolobus solfataricus* AAA protein Sso0909, a homologue of the eukaryotic ESCRT Vps4 ATPase. Biochem Soc Trans 36, 94-98.

Hofmann, K. and Bucher, P. (1995). The FHA domain: aputative nuclear signalling domain found in protein kinasesand transcription factors. Trends Biochem Sci 20, 347–349.

Holland, I. B. and Blight, M. A. (1999). ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. J Mol Biol 293, 381-399.

Holland, I. B., Cole, S.P.C., Kuchler, K. and Higgins, C. F. (2003). ABC Proteins: From Bacteria to Man. Academic Press, London.

Hollenstein, K., Dawson, R. J. and Locher, K. P. (2007). Structure and mechanism of ABC transporter proteins. Curr Opin Struct Biol 17, 412-418.

Horlacher, R., Xavier, K. B., Santos, H., DiRuggiero, J., Kossmann, M. and Boos, W. (1998). Archaeal binding protein-dependent ABC transporter: molecular and biochemical analysis of the trehalose/maltose transport system of the hyperthermophilic archaeon *Thermococcus litoralis*. J Bacteriol 180, 680-689.

Huber, H., Hohn, M. J., Rachel, R., Fuchs, T., Wimmer, V. C. and Stetter, K. O. (2002). A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. Nature 417, 63-67.

Huber, H. and Stetter, K. O. (2000). Bergey's manual of systematic bacteriology. Springer Verlag Electronic Production.

Hubmacher, D., Matzanke, B. F. and Anemuller, S. (2007). Iron-uptake in the Euryarchaeon *Halobacterium salinarum*. Biometals 20, 539-547.

Hurley, J. H. (2008). ESCRT complexes and the biogenesis of multivesicular bodies. Curr Opin Cell Biol 20, 4-11.

Hurley, J. H. and Hanson, P. I. (2010). Membrane budding and scission by the ESCRT machinery: It's all in the neck. Nature 11, 556-566.

Jarrell, K. F. and Albers, S. V. (2012). The archaellum: an old motility structure with a new name. Trends Microbiol 20, 307-312.

Jurgens, G., Lindstrom, K. and Saano, A. (1997). Novel group within the kingdom Crenarchaeota from boreal forest soil. Appl Environ Microbiol 63, 803-805.

Kaidoh K., Miyauchi S., Abe A., Tanabu S., Nara T. and Kamo N. (1996). Rhodamine 123 efflux transporter in *Haloferax volcanii* is induced when cultured under 'metabolic stress' by amino acids: the efflux system resembles that in a doxorubicin-resistant mutant. Biochem J 314, 355-359.

Kaplan, C. D. and Kaplan, J. (2009). Iron acquisition and transcriptional regulation. Chem Rev 109, 4536-4552.

Karner, M. B., DeLong, E. F. and Karl, D. M. (2001). Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature 409, 507-510.

Karpowich, N. K., Huang, H. H., Smith, P. C. and Hunt, J. F. (2003). Crystal structures of the BtuF periplasmic-binding protein for vitamin B12 suggest a functionally important reduction in protein mobility upon ligand binding. J Biol Chem 278, 8429-8434.

Katoh, K., Kuma, K., Toh, H. and Miyata, T. (2005). MAFFTversion 5: improvement in accuracy of multiple sequencealignment. Nucleic Acids Res 33, 511–518.

Kawarabayasi, Y., Hino, Y., Horikawa, H. and other authors (2001). Complete genome sequence of an aerobic thermoacidophilic crenarchaeon, *Sulfolobus tokodaii* strain7. DNA Res 8, 123-140.

Kerr, I. D. (2002). Structure and association of ATP-binding cassette transporter nucleotide-binding domains. Biochim Biophys Acta 1561, 47-64.

Kieffer, C., Skalicky, J. J., Morita, E., De Domenico, I., Ward, D. M., Kaplan, J. and Sundquist, W. I. (2008). Two distinct modes of ESCRT-III recognition are required for VPS4 functions in lysosomal protein targeting and HIV-1 budding. Dev Cell 15, 62-73.

Kim, J. G., Park, S. J., Quan, Z. X. and other authors (2014). Unveiling abundance and distribution of planktonic Bacteria and Archaea in a polynya in Amundsen Sea, Antarctica. Environ Microbiol 16, 1566-1578.

Koerdt, A., Gödeke, J., Berger, J., Thormann, K.M., Albers, S. V. and Godeke, J. (2010). Crenarchaeal biofilm formation under extreme conditions. PLoS ONE 5, e14104.

Kokoeva, M. V., Storch, K. F., Klein, C. and Oesterhelt, D. (2002). A novel mode of sensory transduction in archaea: binding protein-mediated chemotaxis towards osmoprotectants and amino acids. EMBO J 21, 2312-2322.

Koning, S. M., Elferink, M. G., Konings, W. N. and Driessen, A. J. (2001). Cellobiose uptake in the hyperthermophilic archaeon *Pyrococcus furiosus* is mediated by an inducible, high-affinity ABC transporter. J Bacteriol 183, 4979-4984.

Koning, S. M., Albers, S. V., Konings, W. N. and Driessen, A. J. (2002). Sugar transport in (hyper) thermophilic archaea. Res Microbiol 153, 61-67.

Koning, S. M., Konings, W. N. and Driessen, A. J. (2002). Biochemical evidence for the presence of two alpha-glucoside ABC-transport systems in the hyperthermophilic archaeon *Pyrococcus furiosus*. Archaea 1, 19-25.

Koster, W. (2001). ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B12. Res Microbiol 152, 291-301.

Krug, M., Lee, S. J., Diederichs, K., Boos, W. and Welte, W. (2006). Crystal structure of the sugar binding domain of the archaeal transcriptional regulator TrmB. J Biol Chem 281, 10976-10982.

Lalande, M. E., Ling, V. and Miller, R. G. (1981). Hoechst 33342 dye uptake as a probe of membrane permeability changes in mammalian cells. Proc Natl Acad Sci U. S. A. 78, 363-367.

Lassak, K., Neiner, T., Ghosh, A., Klingl, A., Wirth, R. and Albers, S. V. (2012). Molecular analysis of the crenarchaeal flagellum. Mol Microbiol 83, 110-124.

Le, S. Q., Lartillot, N. and Gascuel, O. (2008). Phylogenetic mixture models for proteins. Philos Trans R Soc Lond B Biol Sci 363, 3965-3976.

Lee, S. J., Engelmann, A., Horlacher, R., Qu, Q., Vierke, G., Hebbeln, C., Thomm, M. and Boos, W. (2003). TrmB, a sugar-specific transcriptional regulator of the trehalose/maltose ABC transporter from the hyperthermophilic archaeon *Thermococcus litoralis*. J Biol Chem 278, 983-990.

Lee, S. J., Bohm, A., Krug, M. and Boos, W. (2007). The ABC of binding-protein-dependent transport in Archaea. Trends Microbiol 15, 389-397.

Lepp, P. W., Brinig, M. M., Ouverney, C. C., Palm, K., Armitage, G. C. and Relman, D. A. (2004). Methanogenic Archaea and human periodontal disease. Proc Natl Acad Sci U. S. A. 101, 6176-6181.

Li, Z. and Blissard, G. W. (2012). Cellular VPS4 is required for efficient entry and egress of budded virions of *Autographa californica* multiple nucleopolyhedrovirus. J Virol 86, 459-472.

Lindas, A. C. and Bernander, R. (2013). The cell cycle of archaea. Nat Rev Microbiol 11, 627-638.

Lindas, A. C., Karlsson, E. A., Lindgren, M. T., Ettema, T. J. and Bernander, R. (2008). A unique cell division machinery in the Archaea. Proc Natl Acad Sci U. S. A. 105, 18942-18946.

Lowe, J. and Amos, L. A. (1998). Crystal structure of the bacterial cell-division protein FtsZ. Nature 391, 203-206.

Lubelski, J., Konings, W. N. and Driessen, A. J. (2007). Distribution and physiology of ABC-type transporters contributing to multidrug resistance in bacteria. Microbiol Mol Biol Rev 71, 463-476.

Lundgren, M., Andersson, A., Chen, L., Nilsson, P. and Bernander, R. (2004). Three replication origins in *Sulfolobus* species: synchronous initiation of chromosome replication and asynchronous termination. Proc Natl Acad Sci U. S. A. 101, 7046-7051.

Lundgren, M. and Bernander, R. (2005). Archaeal cell cycle progress. Curr Opin Microbiol 8, 662-668.

Lundgren, M., Malandrin, L., Eriksson, S., Huber, H. and Bernander, R. (2008). Cell cycle characteristics of crenarchaeota: unity among diversity. J Bacteriol 190, 5362-5367.

Makarova, K. S., Yutin, N., Bell, S. D., and Koonin, E. V. (2010). Evolution of diverse cell division and vesicle formationsystems in Archaea. Nat Rev Microbiol 8, 731-741.

Maruyama, H., Shin, M., Oda, T. and other authors (2011). Histone and TK0471/TrmBL2 form a novel heterogeneous genome architecture in the hyperthermophilic archaeon *Thermococcus kodakarensis*. Mol Biol Cell 22, 386-398.

Maaty, W. S., Ortmann, A. C., Dlakić, M., Schulstad, K., Hilmer, J. K., Liepold, L., Weidenheft, B., Khayat, R., Douglas, T., Young, M. J. and Bothner, B. (2006). Characterization of the archaeal thermophile *sulfolobus* turreted icosahedral virus validates an evolutionary link among double-stranded DNA viruses from all domains of life. J Virol 80, 7625-7635.

Makarova, K. S. and Koonin, E. V. (2010). Two new families of the FtsZ-tubulin protein superfamily implicated in membrane remodeling in diverse bacteria and archaea. Biol Direct 5, 33.

Makarova, K. S., Yutin, N., Bell, S. D. and Koonin, E. V. (2010). Evolution of diverse cell division and vesicle formation systems in archaea. Nat Rev Microbiol 8, 731-741.

Mashburn-Warren, L. M. and Whiteley, M. (2006). Special delivery: vesicle trafficking in

prokaryotes. Mol Microbiol 61, 839-846.

Maupin-Furlow, J. A., Humbard, M. A., Kirkland, P. A., Li, W., Reuter, C. J., Wright, A. J. and Zhou, G. (2006). Proteasomes from structure to function: perspectives from Archaea. Curr Top Dev Biol 75, 125-169.

Mayer, F. and Gottschalk, G. (2003). The bacterial cytoskeleton and its putative role in membrane vesicle formation observed in a Gram-positive bacterium producing starch-degrading enzymes. J Mol Microbiol Biotechnol 6, 127-132.

Mietzner, T. A., Tencza, S. B., Adhikari, P., Vaughan, K. G. and Nowalk, A. J. (1998). Fe(III) periplasm-to-cytosol transporters of gram-negative pathogens. Curr Top Microbiol Immunol 225, 113-135.

Moody, J. E., Millen, L., Binns, D., Hunt, J. F. and Thomas, P. J. (2002). Cooperative, ATP-dependent association of the nucleotide binding cassettes during the catalytic cycle of ATP-binding cassette transporters. J Biol Chem 277, 21111-21114.

Moriscot, C., Gribaldo, S., Jault, J. M. and other authors (2011). Crenarchaeal CdvA forms double-helical filaments containing DNA and interacts with ESCRT-III-like CdvB. PloS one 6, e21921.

Morita, E., Colf, L. A., Karren, M. A., Sandrin, V., Rodesch, C. K. and Sundquist, W. I. (2010). Human ESCRT-III and VPS4 proteins are required for centrosome and spindle maintenance. Proc Natl Acad Sci U. S. A. 107, 12889-12894.

Morita, E., Sandrin, V., Chung, H. Y., Morham, S. G., Gygi, S. P., Rodesch, C. K. and Sundquist, W. I. (2007). Human ESCRT and ALIX proteins interact with proteins of the midbody and function in cytokinesis. EMBO J 26, 4215-4227.

Morita, E. and Sundquist, W. I. (2004). Retrovirus budding. Annu Rev Cell Dev Biol 20, 395-425.

Mourez, M., Skouloubris, S., Betton, J. M. and Dassa, E. (1997). Heat shock induction by a misassembled cytoplasmic membrane protein complex in *Escherichia coli*. Mol Microbiol 26, 821-831.

Mukhopadhyay, B., Johnson, E. F. and Wolfe, R. S. (2000). Anovel pH2 control on the expression of flagella in the hyperthermophilic strictly hydrogenotrophic methanarchaeaon *Methanococcus jannaschii*. Proc Natl Acad Sci USA 97, 11522–11527.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67, 593–656.

Ninio, S. and Schuldiner, S. (2003). Characterization of an archaeal multidrug transporter with a unique amino acid composition. J Biol Chem 278, 12000-12005.

Nunoura, T., Takaki, Y., Kakuta, J., Nishi, S., Sugahara, J., Kazama, H., Chee, G. J., Hattori, M., Kanai, A., Atomi, H., Takai, K. and Takami, H. (2011). Insights into the evolution of archaea and eukaryotic protein modifier systems revealed by the genome of a novel archaeal group. Nucleic Acids Res 39:3204-3223.

Obita, T., Saksena, S., Ghazi-Tabatabai, S., Gill, D. J., Perisic, O., Emr, S. D. and Williams, R. L. (2007). Structural basis for selective recognition of ESCRT-III by the AAA ATPase Vps4. Nature 449, 735-739.

Obita, T. (2014). A role for crenarchaeal ESCRT system in cell division. Seikagaku 86, 59-62.

Ochsenreiter, T., Pfeifer, F. and Schleper, C. (2002). Diversity of Archaea in hypersaline environments characterized by molecular-phylogenetic and cultivation studies. Extremophiles 6, 267-274.

Ochsenreiter, T., Selezi, D., Quaiser, A., Bonch-Osmolovskaya, L. and Schleper, C. (2003). Diversity and abundance of Crenarchaeota in terrestrial habitats studied by 16S RNA surveys and real time PCR. Environ Microbiol 5, 787-797.

Olsen, G. J. and Woese, C. R. (1997). Archaeal genomics: an overview. Cell 89, 991-994.

Ortmann, A. C., Brumfield, S. K., Walther, J., McInnerney, K., Brouns, S. J., van de Werken, H. J., Bothner, B., Douglas, T., van de Oost, J. and Young, M. J. (2008). Transcriptome analysis of infection of the archaeon *sulfolobus solfataricus* with sulfolobus turreted icosahedral virus. J Virol 82, 4874-4883.

Panagiotidis, C. H., Boos, W. and Shuman, H. A. (1998). The ATP-binding cassette subunit of the maltose transporter MalK antagonizes MalT, the activator of the *Escherichia coli* mal regulon. Mol Microbiol 30, 535-546.

Peel, S., Macheboeuf, P., Martinelli, N. and Weissenhorn, W. (2011). Divergent pathways lead to ESCRT-III-catalyzed membrane fission. Trends Biochem Sci 36, 199-210.

Pennell, S., Westcott, S., Ortiz-Lombardia, M., Patel, D., Li, J., Nott, T. J. and other authors (2010). Structural and functional analysis of phosphothreonine-dependent FHA domain interactions. Structure 18, 1587-1595.

Pikuta, E. V., Hoover, R. B. and Tang, J. (2007). Microbial extremophiles at the limits of life. Crit Rev Microbiol 33, 183-209.

Podar, M., Makarova, K. S., Graham, D. E., Wolf, Y. I., Koonin, E. V. and Reysenbach, A. L. (2013). Insights into archaeal evolution and symbiosis from the genomes of a nanoarchaeon and its inferred crenarchaeal host from Obsidian Pool, Yellowstone National Park. Biol Direct 22,9.

Pohlschroder, M., Ghosh, A., Tripepi, M. and Albers, S. V. (2011). Archaeal type IV pilus-like structures-evolutionarily conserved prokaryotic surface organelles. Curr OpinMicrobiol 14, 357-363.

Poplawski, A., Gullbrand, B. and Bernander, R. (2000). The ftsZ gene of *haloferax mediterranei*: Sequence, conserved gene order, and visualization of the FtsZ ring. Gene 242, 357-367.

Prangishvili, D., Holz, I., Stieger, E., Nickell, S., Kristjansson, J. K. and Zillig, W. (2000). Sulfolobicins, specific proteinaceous toxins produced by strains of the extremely thermophilic archaeal genus *Sulfolobus*. J Bacteriol 182, 2985-2988.

Prisco, A., Moracci, M., Rossi, M. and Ciaramella, M. (1995). A gene encoding a putative membrane protein homologous to the major facilitator superfamily of transporters maps upstream of the beta-glycosidase gene in the archaeon *Sulfolobus solfataricus*. J Bacteriol 177, 1614-1619.

Punta, M., Coggill, P.C., Eberhardt, R.Y., Mistry, J., Tate, J., Boursnell, C. and other authors (2011). The Pfam protein families database. Nucleic Acids Res 40, 290-301.

Quiocho, F. A. (1990). Atomic structures of periplasmic binding proteins and the high-affinity active transport systems in bacteria. Philos Trans R Soc Lond B Biol Sci 326, 341-351; discussion 351-342.

Quiocho, F. A. and Ledvina, P. S. (1996). Atomic structure and specificity of bacterial periplasmic receptors for active transport and chemotaxis: variation of common themes. Mol Microbiol 20, 17-25.

Raiborg, C. and Stenmark, H. (2009). The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. Nature 458, 445-452.

Reilly, M. S. and Grogan, D. W. (2001). Characterization of intragenic recombination in a

hyperthermophilic archaeon via conjugational DNA exchange. J Bacteriol 183, 2943-2946.

Reno, M. L., Held, N. L., Fields, C. J., Burke, P. V. and Whitaker, R. J. (2009). Biogeography of the *Sulfolobus islandicus* pan-genome. Proc Natl Acad Sci U. S. A. 106, 8605-8610.

Reysenbach, A. L., Ehringer, M. and Hershberger, K. (2000). Microbial diversity at 83 degrees C in Calcite Springs, Yellowstone National Park: another environment where the Aquificales and "Korarchaeota" coexist. Extremophiles 4, 61-67.

Reysenbach, A. L., Liu, Y., Banta, A. B., Beveridge, T. J., Kirshtein, J. D., Schouten, S., Tivey, M. K., Von Damm, K. L. and Voytek, M. A. (2006). A ubiquitous thermoacidophilic archaeon from deep-sea hydrothermal vents. Nature 442, 444-447.

Rodrigues, M. L., Nimrichter, L., Oliveira, D. L. and other authors (2007). Vesicular polysaccharide export in Cryptococcus neoformans is a eukaryotic solution to the problem of fungal trans-cell wall transport. Eukaryot Cell 6, 48-59.

Rodriguez, G. M., Voskuil, M. I., Gold, B., Schoolnik, G. K. and Smith, I. (2002). *ideR*, An essential gene in *Mycobacterium tuberculosis*: role of IdeR in iron-dependent gene expression, iron metabolism, and oxidative stress response. Infect Immun 70, 3371-3381.

Roldán, M. D., Pérez-Reinado, E., Castillo, F. and Moreno-Vivián, C. (2008). Reduction of polynitroaromatic compounds: the bacterial nitroreductases. FEMS Microbiol Rev 32, 474-500.

Rudolph, J. and Oesterhelt, D. (1996). Deletion analysis of the *che* operon in the archaeon *Halobacterium salinarium*. J Mol Biol 258, 548-554.

Rudolph, J., Tolliday, N., Schmitt, C., Schuster, S. C. and Oesterhelt, D. (1995). Phosphorylation in halobacterial signal transduction. Eur Mol Biol Organ J 14, 4249-4257.

Ruiz, J. (2003). Mechanisms of resistance to quinolones: Target alterations, decreased accumulation and DNA gyrase protection. J Antimicrob Chemother 51, 1109-1117.

Sadler, J. E., Shelton-Inloes, B. B., Sorace, J. M., Harlan, J. M., Titani, K. and Davie, E. W. (1985). Cloning and characterization of two cDNAs coding for human von Willebrand factor. Proc Natl Acad Sci USA 82, 6394-6398.

Saksena, S., Sun, J., Chu, T. and Emr, S. D. (2007). ESCRTing proteins in the endocytic pathway. Trends Biochem Sci 32, 561-573.

Samson, R. Y., Obita, T., Freund, S. M., Williams, R. L. and Bell, S. D. (2008). A role for the ESCRT system in cell division in archaea. Science 322, 1710-1713.

Samson, R. Y., Obita, T., Hodgson, B., Shaw, M. K., Chong, P. L., Williams, R. L. and Bell, S. D. (2011). Molecular and structural basis of ESCRT-III recruitment to membranes during archaeal cell division. Mol Cell 41, 186-196.

Saurin, W., Koster, W. and Dassa, E. (1994). Bacterial binding protein-dependent permeases: characterization of distinctive signatures for functionally related integral cytoplasmic membrane proteins. Mol Microbiol 12, 993-1004.

Schleper, C., Holz, I., Janekovic, D., Murphy, J. and Zillig, W. (1995). A multicopy plasmid of the extremely thermophilic archaeon *Sulfolobus* effects its transfer to recipients by mating. J Bacteriol 177, 4417-4426.

Schleper, C., Puehler, G., Holz, I., Gambacorta, A., Janekovic, D., Santarius, U., Klenk, H. P. and Zillig, W. (1995). *Picrophilus* gen. nov., fam. nov.: a novel aerobic, heterotrophic, thermoacidop -hilic genus and family comprising archaea capable of growth around pH 0. J Bacteriol 177, 7050-7059.

Schlesner, M., Miller, A., Streif, S., Staudinger, W. F., Müller, J., Scheffer, B. and other authors (2009)

Identification of Archaea specific chemotaxis proteins which interact with the flagellar apparatus. BMC Microbiol 9, 56.

Schryvers, A. B. and Stojiljkovic, I. (1999). Iron acquisition systems in the pathogenic *Neisseria*. Mol Microbiol 32, 1117-1123.

She, Q., Singh, R. K., Confalonieri, F. and other authors (2001). The complete genome of the crenarchaeon *Sulfolobus solfataricus* P2. Proc Natl Acad Sci U. S. A. 98, 7835-7840.

Shivvers D. W. and T. D. Brock. (1973). Oxidation of elemental sulfur by *Sulfolobus acidocaldarius*. J Bacteriol 114, 706-10.

Smith, P. C., Karpowich, N., Millen, L., Moody, J. E., Rosen, J., Thomas, P. J. and Hunt, J. F. (2002). ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimer. Mol Cell 10, 139-149.

Snyder, J. C., Samson, R. Y., Brumfield, S. K., Bell, S. D. and Young, M. J. (2013). Functional interplay between avirus and the ESCRT machinery in Archaea. PNAS 110, 10783-10787.

Soler, N., Marguet, E., Verbavatz, J. M. and Forterre, P. (2008). Virus-like vesicles and extracellular DNA produced by hyperthermophilic archaea of the order *Thermococcales*. Res Microbiol 159, 390-399.

Spiro, T. G. and P. Saltman. (1969). Polynuclear complexes of iron and their biological implications. Struct Bonding 6, 116-156.

Stamatakis, A., Ludwig, T. and Meier, H. (2005). RAxML-III: a fast program for maximum likely hood-based inference of large phylogenetic trees. Bioinformatics 21, 456-463.

Stetter, K. O. (2006). Hyperthermophiles in the history of life. Philosophical transactions of the Royal Society of London Series B 361, 1837-1842; discussion 1842-1833.

Stetter, K. O. (2006). History of discovery of the first hyperthermophiles. Extremophiles 10, 357-362.

Stetter, K. O. (2013). A brief history of the discovery of hyperthermophilic life. Biochem Soc Trans 41, 416-420.

Stuchell-Brereton, M. D., Skalicky, J. J., Kieffer, C., Karren, M. A., Ghaffarian, S. and Sundquist, W. I. (2007). ESCRT-III recognition by VPS4 ATPases. Nature 449, 740-744.

Sumper, M., Berg, E., Mengele, R. and Strobel, I. (1990). Primary structure and glycosylation of the S-layer protein of *Haloferax volcanii*. J Bacteriol 172, 7111-7118.

Suzuki, T., Iwasaki, T., Uzawa, T., Hara, K., Nemoto, N., Kon, T., Ueki, T., Yamagishi, A. and Oshima, T. (2002). *Sulfolobus tokodaii* sp. nov. (f. *Sulfolobus* sp. strain 7), a new member of the genus *Sulfolobus* isolated from Beppu Hot Springs, Japan. Extremophiles 6, 39-44.

Szabo, Z., Sani, M., Groeneveld, M., Zolghadr, B., Schelert, J., Albers, S. V., Blum, P., Boekema, E. J. and Driessen, A. J. (2007). Flagellar motility and structure in the hyperthermoacidophilic archaeon *Sulfolobus solfataricus*. J Bacteriol 189, 4305-4309.

Tuckwell, D. (1999). Evolution of von Willebrand factor A (VWA) domains. Biochem Soc Trans 27, 835–840.

Van den Berg van Saparoea, H. B., Lubelski, J., van Merkerk, R. and Driessen, A. J. (2005). Proton motive force-dependent Hoechst 33342 transport by the ABC transporter LmrA of *Lactococcus lactis*. Biochemistry 44, 16931-1698.

van de Vossenberg, J. L., Driessen, A. J. and Konings, W. N. (1998). The essence of being extremophilic: the role of the unique archaeal membrane lipids. Extremophiles 2, 163-170.

Vasil, M. L. and Ochsner, U. A. (1999). The response of Pseudomonas aeruginosa to iron: genetics,

biochemistry and virulence. Mol Microbiol 34, 399-413.

Verdon, G., Albers, S. V., Dijkstra, B. W., Driessen, A. J. and Thunnissen, A. M. (2003). Crystal structures of the ATPase subunit of the glucose ABC transporter from *Sulfolobus solfataricus*: nucleotide-free and nucleotide-bound conformations. J Mol Biol 330, 343-358.

Wagner, M., Berkner, S., Ajon, M., Driessen, A. J., Lipps, G. and Albers, S. V. (2009). Expanding and understanding the genetic toolbox of the hyperthermophilic genus *Sulfolobus*. Biochem Soc Trans 37, 97-101.

Wagner, M., Wagner, A., Ma, X., Kort, J. C., Ghosh, A., Rauch, B., Siebers, B. and Albers, S. V. (2014). Investigation of the malE promoter and MalR, a positive regulator of the maltose regulon, for an improved expression system in *Sulfolobus acidocaldarius*. Appl Environ Microbiol 80, 1072-1081.

Wagner, M., Van Wolferen, M., Wagner, A., Lassak, K., Meyer, B. H., Reimann, J., and Albers, S. V. (2012). Versatile genetic tool box for the crenarchaeote *Sulfolobus acidocaldarius*. Front Microbiol 3, 214.

Walker, J. E., Saraste, M., Runswick, M. J. and Gay, N. J. (1982). Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J 1, 945-951.

Wandersman, C. and Stojiljkovic, I. (2000). Bacterial heme sources: the role of heme, hemoprotein receptors and hemophores. Curr Opin Microbiol 3, 215-220.

Wang, B., Yang, S. F., Zhang, L. and He, Z. G. (2010). Archaeal eukaryote-like serine/threonine protein kinase interacts with and phosphorylates a forkhead-associateddomain-containing protein. J Bacteriol 192, 1956-1964.

Wang, X. and Lutkenhaus, J. (1996). FtsZ ring: The eubacterial division apparatus conserved in archaebacteria. Mol Microbiol 21, 313-319.

Weinberg, E. D. (1997). The *Lactobacillus* anomaly: total iron abstinence. Perspect Biol Med 40, 578-583.

Welch, M., Oosawa, K., Aizawa, S. and Eisenbach, M. (1993). Phosphorylation-dependent binding of a signal moleculeto the flagellar switch of bacteria. Proc Natl Acad Sci USA 90, 8787-8791.

Whittaker, C. A. and Hynes, R. O. (2002). Distribution and evolution of von Willebrand/integrin A domains: widely dispersed domains with roles in cell adhesion and elsewhere. Mol Biol Cell 13, 3369-3387.

Whitaker, R. J., Grogan, D. W. and Taylor, J. W. (2005). Recombination shapes the natural population structure of the hyperthermophilic archaeon *Sulfolobus islandicus*. Mol Biol Evol 22, 2354-2361.

Williams, R. L. and Urbe, S. (2007). The emerging shape of the ESCRT machinery. Nature 8, 355-368.

Woese, C. R. and Fox, G. E. (1977). Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc Natl Acad Sci U. S. A. 74, 5088-5090.

Woese, C. R. (1987). Bacterial evolution. Microbiol Rev 51, 221-271.

Woese, C. R., Kandler, O. and Wheelis, M. L. (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci U. S. A. 87, 4576-4579.

Wollert, T., Yang, D., Ren, X., Lee, H. H., Im, Y. J. and Hurley, J. H. (2009). The ESCRT machinery at a glance. J Cell Sci 122, 2163-2166.

Worthington, P., Hoang, V., Perez-Pomares, F. and Blum, P. (2003). Targeted disruption of the alpha-amylase gene in the hyperthermophilic archaeon *Sulfolobus solfataricus*. J Bacteriol 185, 482-488.

Xavier, K. B., Martins, L. O., Peist, R., Kossmann, M., Boos, W. and Santos, H. (1996). High-affinity maltose/trehalose transport system in the hyperthermophilic archaeon *Thermococcus litoralis*. J Bacteriol 178, 4773-4777.

Yen, M. R., Chen, J. S., Marquez, J. L., Sun, E. I. and Saier, M. H. (2010). Multidrug resistance: phylogenetic characterization of superfamilies of secondary carriers that include drug exporters. Meth Mol Biol 637, 47-64.

Yernool, D., Boudker, O., Jin, Y. and Gouaux, E. (2004). Structure of a glutamate transporter homologue from *Pyrococcus horikoshii*. Nature 431, 811-818.

Yuan, Y. R., Blecker, S., Martsinkevich, O., Millen, L., Thomas, P. J. and Hunt, J. F. (2001). The crystal structure of the MJ0796 ATP-binding cassette. Implications for the structural consequences of ATP hydrolysis in the active site of an ABC transporter. J Biol Chem 276, 32313-32321.

Zgurskaya, H. I. and Nikaido, H. (2000). Multidrug resistance mechanisms: Drug efflux across two membranes. Mol Microbiol 37, 219-225.

Zillig, W., Stetter, K. O., Wunderl, S., Schulz, W., Priess H. and Scholz I. (1980). The *Sulfolobus*-"Caldariella" group: Taxonomy on the basis of the structure of DNA-dependent RNA polymerases. Arch Microbiol 125, 259-269.

Zolghadr, B., Klingl, A., Koerdt, A., Driessen, A. J., Rachel, R. and Albers, S. V. (2010). Appendage -mediated surface adherence of *Sulfolobus solfataricus*. J Bacteriol 192, 104-110.

Zolghadr, B., Weber, S., Szabo, Z., Driessen, A. J. and Albers, S. V. (2007). Identification of a system required for the functional surface localization of sugar binding proteins with class III signal peptides in *Sulfolobus solfataricus*. Mol Microbiol 64, 795-806.

# ACKNOWLEDGEMENTS

Finally, it comes to the end… It took 4 and half years to finish my PhD with hard work of days and nights. It's time not only to say "Goodbye", but also "Thank You". I would like to express my gratitude to a number of people who made my PhD study such an enjoyable journey.

My deepest gratitude goes first and foremost to Professor Arnold Driessen, my supervisor. Thank you for offering me a PhD position, it was a pleasure to work in the Molecular Microbiology group. I would like to thank you for your constant encouragements, patience and guidance all these years. You have helped me a lot with completing this thesis. Without your consistent and illuminating instructions, this thesis could not have reached its present form. Thank you also for all the advice in the most difficult moments of my PhD. Thank you for being much more than a supervisor! I really appreciate it!

Albers Sonja, thank you for always willing to answer my questions and give me suggestions on experimental approaches. Juke Lolkema and Dirk-Jan Scheffers, thank you for your valuable suggestions during the group meetings. Janny, Greetje, Jeroen, Susan, thanks for your technical support in the lab. To Bea, Manon, thanks for arranging official matters. I am also greatly indebted to Arjo and Mieke for discussions on isotope-related and some other issues.

I'm so happy to meet so many friendly people during my stay in Groningen.

I would like to thank members of Archaea team: Gosia, thanks for your daily supervision in the initial stage of my research and making our group like a family; Olha, you are the coolest photographer I have known in scientific field and thanks for giving me a lovely turtle! I also would like to thank Samta, for your valuable comments and suggestions during the meetings, best wishes to you and Ilja. Antonella, ciao, hope you donot feel alone when the other group member is gone. Stephan, you are so cool, a tall guy with long blond hair who refuses to use cell phone, talking with you was always so interesting.

I would also like to thank colleagues from MolMic group for making a nice and scientifically stimulating atmosphere. Special thanks go to Jan-pieter, Jeanine, Oleksander, Stefan, Jelger, Erik, Elke, Reto, Lose, Fabiola and Andy, for your supports, helps, interesting and stimulating discussions. Also thank Intan, Irfan, Ali, inshallah. Menno, thank you for translating the summary and the scope of this thesis into Dutch; Jolanda for designing the layout of the cover.

Czeszc, Polish girls! At beginning, I shared the office with Agata, Magda and Gosia, seemed you girls talked about "science" all the time in Polish, and then, Ewa and more polish people joined. That's why my English could not get improved at first (I am joking:). Ewa, I can always hear your laughters, if not yours, it is Ana's.

Nightwatchers: Arsalan, Rustem, Tomec, Marta, we always showed up during the midnights in the lab and disappeared next mornings. It was nice to have your company!

Argentineans: Ola! Lito, your passions, enthusiasms and sharp minds for science showed me what a real scientist is. Pablo, we had great times in Groningen, watching football game and having dinners together. Congratulations on your baby boy!

I'm grateful to all former and current members of Molecular Microbiology group. All the jokes, coffee breaks, smoking times, cakes, borrels and "after holidays" sweets left in the Secretary Corner, you guys made such a nice atmosphere in the MolMic group. I had really good times in Groningen. Thanks to the ones who already left the group: Alexej, Hein, Manfred, Luliia, Marko and Niels. To the current group members: Anne Bart, Danae Angeles, Amalina, Marten, Sabrina,

Ciprian and Fernando. Also Chinese colleagues: Yanping, Min, Liang Zhou, Dongdong Mu, Xiaoguang Du, Xiangfeng Meng.

Special thanks for my paranimfen: Ana, you can always tell interesting stories and show me funny pictures, you are such an amazing girl! Hyun Yong, a quiet gentle man who gives me lots of help. Thank you for the delicious Korea food that your wife made! You are a friend that I can trust and share happiness with.

Last but not least, my thanks would go to my beloved family for their loving considerations and great confidence in me through all these years and I thank Miao simply for having sprinkled my life with stardust, thanks for accompanying with me.

# LIST OF PUBLICATIONS

Nuan Yang, Arnold J. M. Driessen (2014). Deletion of cdvB paralogous genes of *Sulfolobus acidocaldarius* impairs cell division. Extremophiles 18:331–339

Nuan Yang, Arnold J. M. Driessen (2015). The saci_2123 gene of the hyperthermoacidophile *Sulfolobus acidocaldarius* encodes an ATP-binding cassette multidrug transporter. Extremophiles 19:101-108

Julia Reimann, Kerstin Lassak, Sunia Khadouma, Thijs J. G. Ettema, Nuan Yang, Arnold J. M. Driessen, Andreas Klingl and Sonja-Verena Albers (2012). Regulation of archaella expression by the FHA and Von Willebrand domain-containing proteins ArnA and ArnB in *Sulfolobus acidocaldarius.* Molecular Microbiology 86:24–36

Nuan Yang, Arnold J. M. Driessen. Binding protein-dependent iron-uptake in the crenarchaeon *Sulfolobus acidocaldarius*. In preparation